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Development of vaccine conjugates based on Dengue virus using a staphylococcal immune evasion protein

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Award date:
2017

Awarding institution:
University of Bath

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A thesis submitted for the degree of Master of Philosophy

(University of Bath)

Department of Biology and Biochemistry

2017

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Acknowledgments

I would like to express my appreciation to my supervisor professor Jean van den Elsen for giving me the opportunity to be a member of his research group and for all help which was given to me by him. I extend my thanks to Prof Sue Wonnacott for her advice and patience. Special thanks to Dr Mareike Posner for her time, patience and support and also thanks for Dr. Abhishek Upadhyay.

My gratitude also goes to Marjorie Gibbon, Chase, and Omar, there are not enough words to describe their friendly behaviour.

Abstract

Dengue viruses (DENV) are members of the *Flavivirus* family and are thought to infect up to 400 million people per annum and have the potential to lead to large epidemics with subsequent human and economic consequences. The 2 current vaccines being developed are at the clinical trial stage and focus on the induction of neutralising antibodies but there are at least 5 different serotypes of the virus and it appears that induction of immunity to any one serotype will not subsequently provide cross protection to the others. The purpose of this project is to develop an immunisation strategy that will provide protection across all serotypes. A common antigen expressed by all serotypes is designated NS1 which is a non-structural protein expressed on the surface of infected cells. However, on its own NS1 is poorly immunogenic so the intention here is to develop a vector system that combines the expression of NS1 epitopes with those of a highly immunogenic protein derived from *Staphylococcus aureus* the Staphylococcal immunoglobulin-binding protein, Sbi. Sbi is composed of a number of separate domains each of which has characteristic interactive properties with C3. Previous studies have shown that Sbi N-terminal domain IV (Sbi-IV) binds to C3 and its proteolytic fragments, however, the Sbi domains III and IV are essential for fluid phase consumption of C3 activating via the alternative complement pathway. In this project the domains III and IV of Sbi have been cloned and expressed in *E. coli* and conjugated to NS1. These can bind to complement C3, causing cleavage, and lead to the generation of C3d which is able to crosslink binding between the NS1 antigen and complement receptor 2 (CR2) present on phagocytic cells and B cells to greatly enhance a TH2 (antibody) mediated response and ultimately, the generation of memory TH2 cells to provide expansion of B cells (plasma cells) producing DENV specific neutralising antibodies.

List of Abbreviations

AMP	Ampicillin
APS	Ammonium persulphate
IPTG	Isopropyl- β-D-thiogalactoside
LB	Luria broth
CBB	Coomassie Brilliant Blue
CDS	Coomassie de-staining solution
E.coli	*Escherichia coli*
WIESLAB®	Enzyme immunoassay for assessment of Complement functional activity
CP	Classical pathway of complement system
AP	Alternative pathway of complement system
LP	Lectin pathway of complement system
DENF	Dengue Fever
DENV	Dengue Virus
NS1	Non-structural protein of Dengue Virus
Sbi	Staphylococcal immunoglobulin-binding protein
C3	Complement component 3
CR2	complement receptor 2
BL21	competent *E. coli* cells
RT	Room temperature
CD	Circular Dichroism
OD	Optical density
DMSO	Dimethyl sulfoxide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
mRNA	Messenger RNA
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TEMED	N,N,N',N, tetramethylenediamine

PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
PRRs	Pathogen recognition receptors
PAMPs	Pathogen-associated molecular patterns
LTA	Lipoteichoic acid
LPS	Lipopolysaccaride
MBL	Mannose Binding Lectin
MASPs	Mannose binding lectin Associated Serine Protease
FD	Factor D
FB	Factor D
MAC	Membrane Attack Complex
PNH	Paroxysmal Nocturnal Hemoglobinuria
aHUS	atypical Haemolytic Uremic Syndrome
AMD	Age-related Macular Degeneration
FDC	follicular dendritic cells
BCR	B cell surface receptors
Abs	Antibodies
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
LB	Luria-Bertani
DDT	DL-Dithiothreitol
PBS	Phosphate buffered saline
TBS	Tris-buffered saline
PEG	polyethylene glycol

Chapter one: Introduction

1 Introduction

Dengue is a viral disease transmitted and spread largely by the mosquito genus *Aedes aegypti* (and to some extent *A. Albopictus*) which is easily recognised by the silvery white pattern on its scales and has become endemic in many tropical areas of Africa and Asia. The virus is largely spread by female mosquitoes when they prey on large mammals and humans as they require a blood meal to produce eggs. The virus is normally present in the salivary glands of the mosquito, so upon biting the host the virus is transmitted and then propagates in the host (1, 2).

The mosquito – human - mosquito cycle of transmission leads to a typical cycle being: infected mosquito bite leading to (after about 4 days) acute viremia in the host which usually lasts for about 5 days and then the development of symptoms of dengue which can last for a week. The mosquito vectors become infected when they feed on humans during the four/five-day period of viraemia. The virus passes from the mosquito intestinal tract to the salivary glands after an extrinsic incubation period, a process that takes approximately 10 days and is most rapid at high ambient temperatures (3). Mosquito bites after the extrinsic incubation period result in infection, which might be promoted by mosquito salivary proteins (4).

Although the many cases of Dengue fever are misclassified or indeed under-reported it is generally recognised that there are as many as 400 million Dengue infections per annum of which about a quarter are shown as clinically relevant infections. From these figures published by the World Health Organisation (WHO) it has been estimated that up to half a million people require hospitalisation because of Dengue fever per annum (5). Following infection there are no specific drug treatments available and patient survival depends largely on maintaining patient homeostasis through transfusion of fluids.

The Dengue virus is a positive sense single stranded RNA virus with 10 encoded genes. The genome is translated as a single polypeptide which is subsequently cleaved into the 10 encoded proteins. Of these 10, three are capsid (C), envelope (E)

and membrane (M) proteins and the other 7 are non-structural proteins that are important in viral replication and assembly (Figure 1)(6, 7).

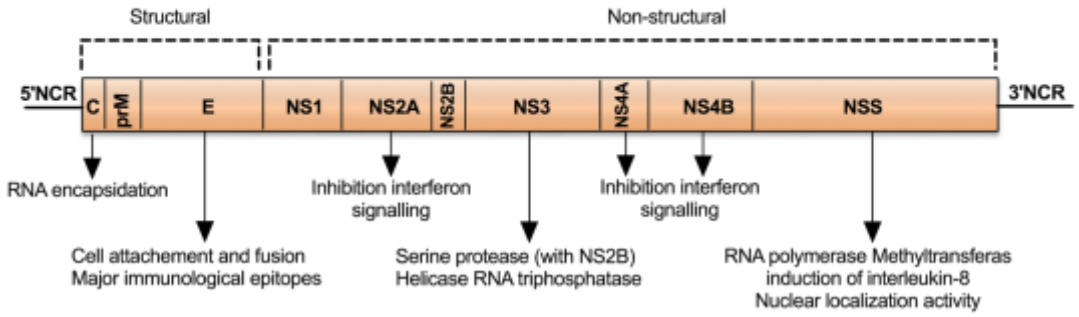

Figure 1: **Dengue virus genome diagram shows the capsid (C), envelope (E) and membrane (M) proteins and the other 7 are non-structural proteins**

Over a period of time it has become clear that there are a number of different serotypes of Dengue virus defined by variations in the structure of vial envelope proteins and these have been defined as DEN-1, DEN-2, DEN-3 and DEN-4. The serotype classification is largely based on E envelope gene sequence data (1485 bp) defining the 4 distinct serotypes but advanced sequencing techniques are likely to uncover even greater variations including the recently described 5 serotype (DENV-5). DENV-2 is associated with most severe disease progression with a greater propensity for transmissibility, increased virulence and faster replication (8, 9).

Recovery from infection by one serotype provides lifelong immunity against that particular serotype. However, cross-immunity to the other serotypes after recovery is only partial and temporary. Subsequent infections by other serotypes increases the risk of developing severe dengue fever (10).

It has been proposed that the process of infection through dengue virus initial exposure and uptake by dendritic cells in the skin proceeds via ICAM3-grabbing non-integrin (DC-SIGN) receptors of immature dendritic cells (11) which then migrate and mature in regional lymph nodes promoting further presentation of viral epitopes and thus enhancement of disease progression through the development of inflammation and the recruitment of mature T cells. This phenomenon involves the development and maturation of both type 1 and type 2 immune responses and leads

to the activation of monocytes and macrophages which can participate in so called antibody-dependent enhancement (ADE) (12) (13). ADE is particularly enhanced in the presence of phagocytes which naturally take up immune complexes (via Fc receptors) formed from DENVs and non-neutralizing antibodies. There is a suggestion that the non-neutralizing antibodies found in recovering patients result from either previous heterotypic dengue infections or from low concentrations of dengue antibodies of maternal origin found in infant sera (14)). Thus the co-existence of four DENV serotypes in a given population might allow the augmentation of population based immune protection by the ADE phenomenon (15).

1.1 Current treatment

The protocols used for dealing with severe dengue infection were developed as long ago as the 1960's following an outbreak affecting many children in Thailand (16) and have been gradually refined since then. Accurate diagnosis is vitally important in recognising the early signs of disease onset such as a rising haemocrit and falling platelet levels as well as abdominal pain and vomiting (although these can also be signs of non-dengue related disease) (17). ELISA and real-time PCR tests can also aid accurate diagnosis but these are often impractical in real-life settings. In practice, treatment is largely through management of fluids but in severe disease this is difficult as fluid overload can be just as dangerous as too little fluids (18).

There have also been considerable efforts applied to the development of anti-dengue drugs especially those aimed at the inhibition of viral entry which is mediated by the dengue virus E protein and there has been some promising early results (19). Other potential targets are the proteins NS3 and NS5 which have a pivotal role in cell replication but these are very much still at the developmental rather than clinical trial phase (20).

As current treatment regimes are often quite ineffective, vaccination is one of the main approaches being undertaken to try and prevent infection in the human host. However, this is complicated because of the large number of different serotypes of the virus. It has been shown that immunisation with one of the 4 well recognised

serotypes does not confer protection against the others and to compound the problem it has been well described that following immunisation of one serotype, infection with one of the other viral serotypes exacerbates disease progression which is referred to as immune enhancement of the disease (10).

Vaccine design most usually involves inducing the production of 'neutralising antibodies'. Neutralising antibodies are an important aspect of preventing repeated infection to microorganisms. They are usually produced towards the later stages of an infection and are generally specific for the receptor ligands on the microorganism preventing binding to targeted host tissues. In the case of DENV infections where up to 4 or 5 separate and discrete subtypes have been shown to exist, it has been suggested that the partial cross-reactivity of antibodies induced by an earlier infection of one DENV subtype may not be of sufficient avidity to neutralise a secondary infection with a discrete subtype and rather than give protection against infection may provide a degree of opsinisation enhancing uptake of the virus into antigen presenting cells (e.g. macrophages) leading to increased spread throughout the body and enhanced viral replication – so called antibody-dependent enhancement (ADE) (12, 21).

So currently there are no really effective vaccines against Dengue virus but a number of approaches with the backing of WHO are being tested. The most advanced trials are with attenuated forms of the virus normally given in combinations of serotypes. As mentioned above, one of the main concerns about inducing immunity to Dengue following immunisation is the problem of disease enhancement and it has thought that by immunising simultaneously with multiple sub-types of the virus this could be overcome. However, preliminary evidence suggests that even this approach may not circumvent the problem of disease enhancement caused by the production of neutralising antibodies produced with low affinity and sub-optimal titres (22, 23).

As discussed earlier, current vaccine strategies are not effective because of the broad range of serotypes existing in wild populations and, indeed, may actually lead to greater pathogenesis because of disease enhancement. Thus targeting the non-

structural and highly conserved protein for vaccine development could provide an important new approach to this serious health problem.

1.2 Innate immunity

The immune system consists of 2 separate 'arms' - innate and adaptive immunity. While adaptive immunity has the property of being highly specific and also possesses 'memory' of previous infections it takes a long period to become effective. Thus a sudden highly virulent infection can lead to death before adaptive immunity is able to neutralise the pathogen by recruitment of T-lymphocytes, B-lymphocytes and humoral components that include immunoglobulins and cytokines (24, 25). This is why the innate immune response plays a vital role as the 'first line' of immune defence (Figure 2).

The innate immune system on the other hand is able to respond to pathogens and recognise pathogenic organisms using pathogen recognition receptors (PRRs) which are able to bind to microbial associated molecules or molecular 'patterns' present only on micro-organisms (pathogen-associated molecular patterns (PAMPs), not present in human tissues (26). Examples of PAMPs include Lipoteichoic acid (LTA) of Gram-positive bacteria, Lipopolysaccaride (LPS) of Gram-negative bacteria, mannan in yeasts, microbial glycolipids and RNA and DNA from viruses. The critical element of the innate immune response is its ability to very quickly mount an effective defence against viruses and bacteria. The importance of innate immunity to protection from microbial infection is illustrated by the fact that there are only very rare cases of people found with poorly effective innate immune responses (27-29).

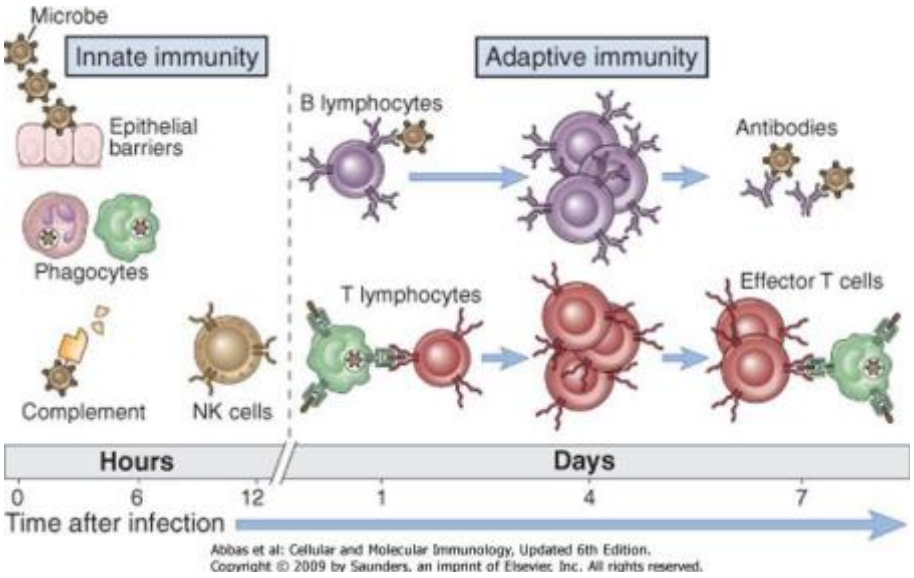

Figure 2: **Schematic representation of the roles of innate and adaptive immunity in responding to infectious disease (30)**

1.3 The complement system

The complement system is a crucial arm of the innate immune defence. It consists of more than 30 proteins in serum, tissue fluids and on cell surfaces (31). Hepatocytes are considered to be the primary site for biosynthesis of complement proteins although extra-hepatic sources of complement protein synthesis have also been reported which include monocytes, macrophages, pulmonary epithelial cells, osteoblasts, adipose tissue and myoblasts (32). There are three different pathways of complement activation: namely the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (LP) (igure 3). Complement proteases exist in serum as inactive zymogens that, upon activation, develop into a cascade of enzymatic reactions involving the assembly of proteolytic complexes and the conversion of complement-zymogens from their inactive state to an enzymatically active state (30). Upon activation, all of the three pathways converge at the C3 cleavage stage which is the most crucial step in complement activation.

The classical pathway is mainly initiated by the binding of C1 to antigen-antibody complexes (33). Clusters of IgG1, IgG2, IgG3 and IgM molecules on antigen surfaces lead to binding of the classical pathway recognition molecule C1q. This binding then leads to activation of the associated serine protease C1r. Upon activation, C1r cleaves and activates C1s which in turn cleaves C4 into C4a and C4b. In a second cleavage step, active C1s cleaves C4-bound C2 into C2a and C2b. C4b and C2a stay bound to the surface to form the classical pathway C3 convertase, C4bC2a while the C4a and C2b are released into the fluid phase (34-36). In addition to the antigen-antibody route of classical pathway activation, some microorganisms and compounds have been found to directly initiate the activation of the classical pathway including C-reactive protein, Gram-negative bacteria and viral envelopes (37).

Another route of complement activation is the lectin pathway. This pathway is initiated by the binding of lectin carbohydrate recognition molecules to pathogen

surfaces. In the human, more than five different recognition molecules have been identified so far, including Mannose Binding Lectin (MBL), ficolins (L-ficolin [also known as ficolin-1], M-ficolin [also known as ficolin-2], H-ficolin [also known as ficolin-1]) and Collectin-11 (CL-11). The pathway involves three serine proteases i.e. MASP-1, MASP-2 and MASP-3 for Mannose binding lectin Associated Serine Protease (38-40).

The binding of carbohydrate recognition molecules to their target leads to the activation of its associated serine protease. Similarly to the classical pathway molecule C1r, MASP-2 cleaves both C4 and C2 generating C4bC2a which is in effect the lectin pathway equivalent of C3 convertase. MASP-1, however, has the ability to cleave C2 but not C4 (41). The most recently discovered MASP, MASP-3, is devoid of enzymatic activity against C2, C3 and C4 (40). However, recent publications have claimed that MASP-1 and MASP-3 have the ability to cleave and activate the alternative pathway effector enzyme factor D (42).

C3 convertase produced by either the classical pathway or the lectin pathway, C4bC2a, cleaves its unique substrate, C3, into two unequally sized fragments, C3a (9 kDa) and C3b (171 kDa). C3b binds to C3 convertase to form C5 convertase, C4bC2a(C3b)n (35, 43).

The alternative complement pathway (AP) plays a crucial role in immune protection and it has been suggested that approximately 80-90% of all complement activation goes via the alternative pathway (44). As shown earlier, the key component of the initiation of complement activation is by the cleavage of C3 to C3b and other cleavage products and this can be initiated through the classical or lectin pathways which in turn can activate the highly effective alternative pathway. In the alternative pathway of complement activation, factor B (FB) binds to a C3b-bound activator surface and undergoes conformational changes exposing a new cleavage site for factor D (FD). FD cleaves C3bB to form the alternative pathway C3 convertase (C3bBb) and the latter cleaves C3 into C3a and C3b (35, 43)

However, there is another route by which C3 can be cleaved using spontaneous hydrolysis of native C3 to produce C3(H_2O). The thio-ester bond of native C3 undergoes hydrolysis and that lead to emerge new binding site of FB which allows binding of FB to form the C3(H_2O)B complex. In turn factor D cleaves factor B in that complex to form C3(H2O)Bb which is a further route to the generation of AP activation (34, 35).

Following C3 activation, a C5 convertase will be formed by the binding one or more molecules of C3b to C3 convertase, C4BC2a(C3b)n or C3bBb(C3b)n, that will activate complement C5 via cleaving it into C5a and C5b. The activated C5 stimulates the assembly of the terminal complement components C6, C7, C8 and C9 with the formation of the so-called Membrane Attack Complex (MAC). MAC has the capacity to insert into target cell membranes with subsequent lead to lysis of these susceptible target cells (45).

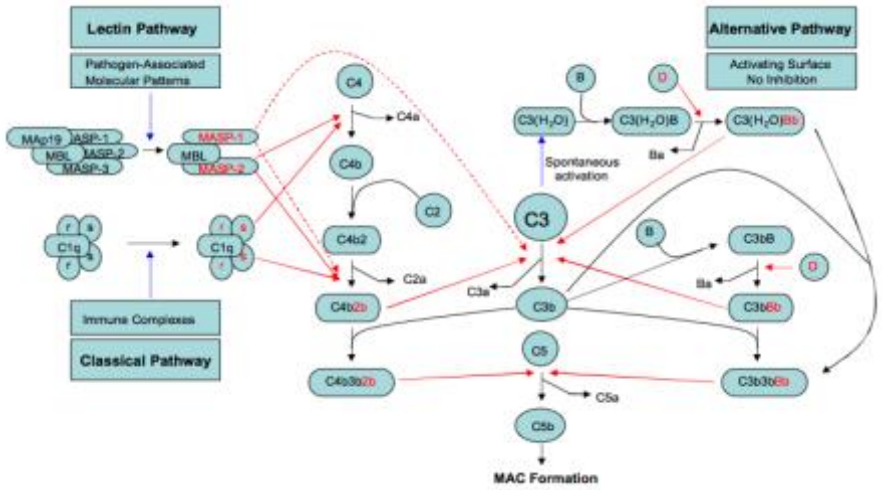

Figure 3: Schematic representation of the complement pathways

The 3 different complement pathways that have different initiating pathways which converge at the C3 cleavage stage which is the most crucial step in complement activation (46).

As a part of the innate immune system, complement protects the body against invading pathogens via one or more of the following mechanisms; recruitment of immune cells, opsonisation and lysis of pathogens via MAC formation (47).

Complement activation leads to a constant release of pro-inflammatory signals, including the anaphylatoxins C3a and C5a, which trigger phagocytosis. C3a and C5a are strong chemoattractants that trigger activation of phagocytes (i.e. monocytes, macrophages and neutrophils) to the site of infection to remove the opsonised cells (48).

In spite of the important and crucial role of the complement system in innate immune defence against infection, complement activation can have a detrimental effect on self-cells and is thus tightly controlled by a number of feedback signals. The contribution of the complement system to the pathophysiology and development of several autoimmune diseases has been well documented and uncontrolled complement activation can also lead to the development of inflammatory diseases such as Paroxysmal Nocturnal Hemoglobinuria (PNH), atypical Haemolytic Uremic Syndrome (aHUS), Age-related Macular Degeneration (AMD), and membranoproliferative glomerulonephritis (49).

Viral infections have the ability to initiate the activation all 3 complement pathways. For instance, C1q, C3b and C4b can bind directly to viron surfaces leading to opsinisation and the recruitment of phagocytic cells and may also directly prevent the interaction of viruses with their appropriate receptors preventing uptake (50). In addition, C3 has been shown to directly inactivate human immunodeficiency virus and the formation of multivalent complexes of viruses, antibodies and complement serve to neutralise viral infectivity. The terminal components of the classical complement pathway (C5a-C9) have also been shown to directly attack viral envelopes leading to pore formation and osmotic lysis and loss of viral viability as has been shown for alphaviruses, coronaviruses, herpesviruses, and retroviruses (51, 52).

During dengue hemorrhagic fever DHF, the complement cascade is also activated and the levels of the complement activation products C3a and C5a correlate with the severity of illness (53). Both soluble and membrane-associated NS1 have been demonstrated to directly activate human complement and the levels of the terminal complement complex (SC5b–9) and plasma NS1 concentration both correlate with disease severity, suggesting a link between the virus, complement activation and the

development of DHF and dengue shock syndrome (DSS) (54). Alternative hypotheses of dengue pathogenesis include the suggestion that secondary T-cell responses are blunted because stimulation of the T-cell memory response results in the production of heterotypic CD4+ and CD8+ cells that have a diminished capacity to neutralise the infection but, at the same time, release inflammatory cytokines that contribute to disease severity (55); this leads to the development/maturation of DENVs with an increased virulence causing more severe disease (56); and, finally, there has been a suggestion that cross-reactivity between the NS1 protein and components of human platelets and endothelial cells leads to the production of cross reactive antibodies that damage these cells increasing the pathological consequences (57).

1.4 Complement C3d and Staphylococcal Sbi protein as an adjuvant strategy

In the cleavage process which produces C3b from C3, another important fragment is produced (C3d), which is the final degradation product of C3. As a natural and powerful opsonin, C3d provides an excellent way of switching on Th2 activation. C3d binds to complement receptor 2 (CR2) that is located on the surface of follicular dendritic cells (FDC), B cells and some subsets of T cells. C3d stimulates antigen presentation by FDCs and helps to maintain B cell memory which in turn can be highly effective in initiating and perpetuating an immune response, thus acting as an adjuvant for any linked antigen (58, 59).

Vaccine development can be focused through different effector pathways of the immune response to establish immunological memory and classical approaches to vaccine design try to induce the production of neutralising antibodies which are effective against viral infections as these prevent entry of the virus (upon later exposure) to its target cells. For this to be successful, it is necessary for the vaccine to initiate a Th2 response which induces antibody mediated immunity. The *Staphylococcus aureus* immune evasion protein Sbi is unique in its ability to interact with components of both the adaptive and innate arms of the immune system. Sbi has 4 domains – Sbi-I and Sbi-II bind IgG and Sbi-IV, on its own, specifically inhibits the alternative pathway of complement activation but when linked to Sbi-III induces a futile consumption of complement (Figure 4).

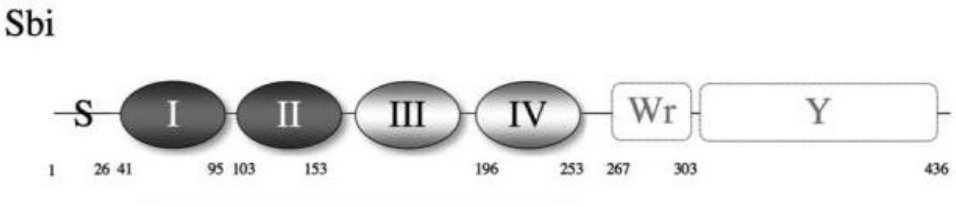

Figure 4: Schematic representation of the domain structure of Sbi taken from (60)

Thus expression of a combination of Sbi domains III and IV has the potential to recruit and activate complement through binding to the C3d component of complement (60). Normally activation of B cells and the initiation of B cell clonal proliferation requires cross-linking of the target antigen epitope with the appropriate B cell surface receptors (BCR) but by combining the antigen with C3d, the combined antigen can directly crosslink the BCR on any one B cell precursor with CR2, greatly enhancing the potential activation of B cells even when these may have a very low precursor frequency. In addition, enhanced expression of C3d can act as a chemical attractant, again improving the activation of Th2 directed immunity (59) (Figure 5).

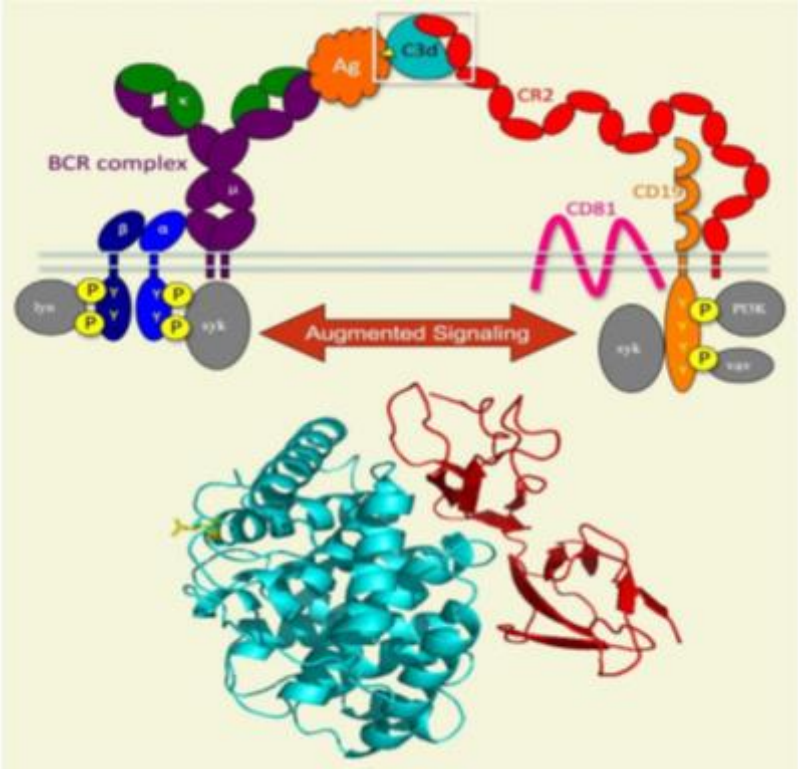

Figure 5: diagrammatic representation of how C3d binding to a target antigen via CR2

This binding can greatly enhance stimulation of B cells and consequently enhance phagocytosis leading to enhanced antibody maturation and long-term memory (61)

1.5 Dengue non-structural protein 1 (NS1) as a vaccine antigen

Given the proven difficulties in developing an effective vaccine against the different serotypes of the Dengue virus, this project sets out a coherent approach which can lead to the induction of neutralising antibodies caused by local complement activation and opsonisation of the antigen by using the complement C3d fragment as a natural adjuvant. Rather than trying to target the numerous antigens present on the various serotypes the project sets out to target the non-structural protein 1 (NS1) of the virus that is highly conserved and is present in all serotypes of DENV (62) thus overcoming the problem of trying to induce a protective response to the numerous subtypes. NS1 is a glycosylated 48kD protein that has been shown to be important not only in viral replication but also seems to play a role in immune evasion (63, 64).

It has also been shown that NS1 is a major target of humoral immunity in patients recovering from disease and is also believed to be involved in DENV pathogenesis. Some authors have suggested the NS1 protein itself (as well as virons) when taken up by endothelial cells in the liver may actually promote and enhance viral infection of the liver cells so this evidence supports the concept that the non-structural viral protein NS1 plays an important role in DENV disease pathogenesis (53, 65). NS1 is also a useful diagnostic marker for the presence of dengue virus using enzyme-linked immunosorbent assays (ELISAs) as it is secreted and circulates in the plasma even at an early stage of viral infection. It has been shown that NS1 can directly bind host components of complement which leads to inhibition of complement activation in solution (66). The immune response to dengue virus (DENV) infection generates high levels of antibodies (Abs) to NS1, particularly in cases of secondary infection (67, 68). It has also been suggested that NS1 may contain epitopes that mimic self-epitopes present on host molecules intensifying the pathogenic effects of DENV infection as seen with DHF and DSS. Increasing evidence for the important role of NS1 in the development of DENV pathogenesis thus enhances the case for focusing attention on NS1 as a target for inducing protective immunity (54, 69, 70).

1.6 Outline of the project objectives

The project therefore set out to express a pGEX-htb construct (combining the Sbi-III-IV and NS1 genes) using the pET28a expression system in BL21 *E. coli*. Possibly because of a lack of glycosylation in the *E. coli* expression system the fusion protein was expressed in inclusion bodies because of poor solubility, so a large part of the project was devoted to investigating the best method to yield a soluble/functional protein which could be characterised by circular dichroism and assessed for its functional activity by complement activation assays *in vitro*.

The key aims of the project were therefore to:

- Express the vector in *E. coli* and purify the expressed protein.
- Characterise the fusion protein biochemically (e.g. sequencing, circular dichroism).
- Assess the function of the fusion protein in complement activation assays and complement C3b deposition.

Chapter Two: Materials and Methods

2 Materials and Methods

2.1 Epression and purification of recombinant Sbi-III-IV-NS1

2.1.1 Cloning

The coding sequence of Sbi domains 3 and 4 was cloned into the pET28a (Kan^r)vector using the *Nhe*I and *BamH*I restriction sites and, in addition, the coding sequence of Dengue virus NS1 was inserted using the *BamH*I and *Xho*I restriction sites to make a plasmid construct that can be expressed in *E. coli*. The recombinant Sbi-III-IV-NS1 carries a N-terminal His-tag for protein purification. (This work was performed by Dr Gyles Cozier). See appendix 1 for the coding sequence.

2.1.2 Expression of Sbi-III-IV-NS1 using E. coli BL21 (DE3)

The *E. coli* BL21 (DE3) strain was used to express the Sbi-III-IV-NS1 fusion protein. Bacteria containing the plasmid were grown overnight in LB medium containing a final concentration of 1 mM of Kanamycin at 37°C. 15ml of the overnight culture (primary culture) were added to 1L of LB medium containing 1 mM. Cells were grown at 37°C with shaking at 180 rpm. The expression of the Sbi-III-IV-NS1 fusion protein was induced at different temperatures i.e. 12°C, 16°C and 25°C during the exponential growth phase (OD_{600}=0.8) in the presence of 0.5mM isopropyl thiogalactoside (IPTG). *E. coli* cells were then centrifuged at 8000*g* for 20 min at different time points including 16h, 12h and 8h after induction. The cell pellet was resuspended in 20ml of HisA buffer (20mM Tris + 300mM NaCl + 20mM imidazole). The re-suspended cells were sonicated on ice 6 times at 80% amplitude for 30 seconds separated by 5 minute intervals and the resulting cell lysate was centrifuged at 60,000*g* for 30min at 4°C.

2.1.3 Purification

The supernatant was collected and the recombinant protein purified by ion exchange chromatography using HiTrap™ HP column (GE Healthcare). The HiTrap™ HP chromatography column was operated by using the AKTA purifier system of GE Healthcare which allows monitoring the progress of purification and measuring the conductivity and UV/Vis absorbance at 280 nm for detecting the proteins. The supernatant was loaded onto a 1ml Hitrap column (GE healthcare) using an AKTA purifier (GE) with a flow rate of between 0.8ml/min and 1.5 ml/min. The loaded column was washed with 5 column volumes of HisA buffer and the bound Sbi-III-IV-NS1 fusion protein was eluted with HisB (20mM Tris, 300mM NaCl, 500mM imidazole) elution buffer.

2.2 Isolation of the Sbi-III-IV-NS1 plasmid DNA

The *E. coli* BL21 (DE3) strain did not express the protein as a soluble protein. In order to transform the Sbi-III-IV-NS1 plasmid into a different bacterial host as another approach to express the required protein, the The pET28a (Kan^r) vector and Sbi-III-IV-NS1 plasmid DNA was isolated and purified using the GeneJET Plasmid Miniprep Kit (Thermo- fisher). Following the manufactures instructions, briefly, 7 ml of the overnight culture was centrifuged for 10 minutes at 2000 g at 4 ^{O}C. The supernatant was discarded and the pellet re-suspended with 250 µl of cell re-suspension solution, mixed by vortex and transferred into a micro centrifuge tube followed by adding 250 µl of cell lysis solution and the tubes were then kept for 2-3 minutes at room temperature (RT). At the end of the incubation, 10 µl of alkaline protease solution was added and mixed and then the micro centrifuge tube was left at RT for 3 minutes. In the next step, 350 µl of neutralisation solution was added and mixed immediately. Samples were spun down at 13,000 g for 10 minutes. The clear supernatant was transferred onto a spin column which has a filter (provided by the kit) and centrifuged for 60 second. The flow through was discard and the column was washed by adding 750 µl of washing buffer to the spin column and the column was centrifuged at 13,000 g for 60 seconds. The flow through was discarded and the column left in a safety cabinet for 15 minutes to remove the ethanol and the plasmid

DNA was eluted in clean tubes by adding 55-65 µl of Nuclease free water and centrifuged at a maximum speed of 13,000 g for 60 seconds and the eluted DNA was stored at -20ºC.

2.3 Expression of Sbi-III-IV using Rosetta ™ 2(DE3)

The pET28a (Kan^r) vector and of the Sbi-III-IV-NS1 plasmid were extracted from the transformed cells using the GeneJET Plasmid kit. The Rosetta™ 2(DE3) competent cells were recovered from -20°C and thawed on ice. 5µl of the extracted plasmid were added to 10µl of the competent cells and mixed gently, then incubated on ice for 20 minutes. After that, cells were heat shocked in a water bath at 42°C for 45 seconds, to allow uptake of the plasmid by bacterial cells, then transferred immediately into ice for another 2 minutes then 900 µl of LB broth was added to the tubes and incubated at 37°C for 1-2 hours with gentle shaking at 180 rpm. Two different volumes; 50 µl and 100 µl were placed onto LB media plates containing 1 mM of kanamycin and chloramphenicol antibiotics (35µg/ml) and then incubated overnight at 37°C. The next day, one colony was picked up using a sterile tip then inoculated in a universal tube containing 10ml LB medium, and incubated at 37°C for overnight with shaking at 180 rpm. For protein expression, the growth culture was transferred to a flask containing 1 L of LB medium containing 1 mM of kanamycin and 35 µg/ml chloramphenicol and the flask was incubated at 37°C with shaking until the OD_{600}=0.8. At this time, protein expression was induced by addition of IPTG to a final concentration of 1mM. Bacteria were harvested after 4, 8, 12 and 18 hours of incubation by centrifugation at 4000 x g, 4ºC, for 20 minutes and the cell pellets were re-suspended in PBS for washing. The cells were then sonicated on ice for 6 times at 55% amplitude for 30 seconds separated by 5 minutes and the cell lysate was centrifuged at 6000 x g for 30 minutes at 4ºC.

2.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to identify the molecular weight of the proteins, samples were resolved by SDS-PAGE (NOVEX).40 µl of the purified protein was mixed with 10 µl of 6X SDS loading dye containing 1% β-mercaptoethanol (reducing conditions) and the mixture was heated at 90°C for few minutes. 25 µl of this mixture were loaded into 12% SDS gel wells. The gels were run at 150 volts for 40 minutes. The gels were then stained with Coomassie Brilliant Blue.

In order to identify the molecular weight and visualize the protein bands, gels were incubated with Coomassie brilliant blue R-250 (Serva)solution with gentle shaking for 20 - 25 minutes and the gel was de-stained by using de-staining solution (30% methanol, 10% acetic acid, 60% H_2O).

2.5 Optimization of the expression and purification of inclusion bodies

2.5.1 Optimization of the expression of recombinant Sbi-III-IV-NS1

To optimize the Sbi-III-IV-NS1 protein expression *E. coli* BL21 (DE3) strain was used. Bacteria containing the plasmid were grown overnight at 37°C with shaking at 180 rpm in LB medium supplemented with 1 mM of kanamycin. 15ml of the primary culture was added to 1L of LB medium containing 1 mM kanamycin. The culture was incubated at 37°C until the OD_{600} reached 0.8. The Sbi-III-IV-NS1 expression was induced by adding IPTG to a final concentration of 1mM. After 4-5 hours of incubation, the bacteria were spun down at 8000 g for 20 minutes. The cell pellet was washed once in 40 ml of PBS and the pellet was then kept at -80 ºC.

2.5.2 Washing and Processing Protocol for Inclusion Bodies

Following protein expression, four buffers were used to process the cell pellet including: lysis buffer, wash buffer 1 (Triton X-100), wash buffer 2 (Urea) and Bug Buster. Firstly, the pellet was recovered from -80 ºC and defrosted on ice. Forty millilitres of lysis buffer (25mM Tris-HCL pH 8.0, 150 mM NaCl, 0.5 mg/ml lysozyme, 1 mM EDTA) were used to dissolve the pellet and a tablet of protease cocktail inhibitor (Roche) was added. The mixture was incubated for 30 minutes at room temperature (RT) with shaking. A mixer buffer (5 mM $MgCl_2$, 5 µg/ml DNAse) was added to the solution and incubated for 15 minutes at RT with shaking followed by sonication (amplitude at 60% 8 to 9 pulses, 30 seconds per pulse with 3 minute between) and then centrifugation at 20,000g for 20 minutes at 4ºC. The lysate was collected and supernatant was discarded. The pellet was then re-suspended in 40 ml of wash buffer 1 (25 mM Tris-HCL pH 8.0, 0.5 M NaCL, 0.5% Triton X-100, 1mM EDTA. pH 8). Two sonication pulses were applied before further purification followed by centrifugation as in the previous step. After discarding the supernatant, wash buffer 2 was used (25mM Tris-HCL pH 8.0, 0.5 M NaCl, 1 mg/ml sodium deoxycholate, 1 M Urea) followed by another round of sonication and the mixture was then centrifuged. The pellet was washed with 40 ml

of 1:10 protein extraction master mix reagent (BugBuster) followed by sonication and spun down. After this step the pellet was dissolved in 10 ml of 25 mM Tris-HCL pH 8.0 buffer. Finally, the suspended protein was distributed into 1ml Eppendorf tubes, centrifuged at 13,000 rpm for 10 minutes and the pellets were stored at -80 ºC.

2.5.3 Inclusion Bodies – Optimizing solubilisation and refolding

After completing washing and purification of the inclusion bodies, samples were dissolved in 50 ml of solubilisation buffer (25 mM Tris-HCL pH 7.5, 8 M Urea with 5 mM DDT at pH 8) and incubated in a water bath at 42ºC for 15 minutes. Subsequently, the suspension was centrifuged to remove any remaining insoluble cell debris. The supernatant was collected and the concentration was adjusted to 1 mg/ml in each experiment to be used for the refolding process as the manufacture instructions.

The refolding process was started by screening the optimal refolding buffer using the QuickFold™ Protein Refolding Kit (Athena) for the Sbi-III-IV-NS1 protein. The kit contains 15 buffers which were screened for their ability to refold the protein. 4 buffers were then chosen to be used for refolding experiments (see Appendix 3 for buffers).

Once the appropriate refolding buffers were determined, 50 ml of the solution containing soluble protein was mixed slowly by drop method with the refolding buffer at 4ºC overnight, using a 50 ml syringe with gentle stirring. The refolded protein was then dialysed against dialysis buffer (25 mM Tris-HCL pH 7.4, 150 mM NaCl) at 4^{o}C overnight.

2.5.4 Purification using immobilized metal affinity chromatography

To facilitate the purification process, the code for six consecutive histidine residues was added to the N-terminus of the Sbi-III-IV-NS1 plasmid insert. The HisGravi Trap column (GE Healthcare) provides a rapid purification of Histidine-tagged proteins. To obtain the maximum purity of the recombinant protein, a solution containing the refolded protein was run through a HisGravi Trap column (GE Healthcare) allowing the solution to flow through the column by gravity. The column was then washed with 20 ml washing buffer (PBS buffer pH 7.4, containing 20 mM imidazole). Finally, the protein was eluted using 5 ml elution buffer (PBS buffer pH 7.4, containing in order 100mM, 200mM, 300mM and 500mM imidazole) to define the optimal elution concentration of imidazole.

2.6 Measurement of the protein concentration

To measure the Sbi-III-IV-NS1 protein concentration, the Nano drop 2000c spectrophotometer (Thermo Scientific) was used, using an extinction coefficient of $16.29 M^{-1} cm^{-1}$.

2.7 Complement activation assay assessment using the Wielisa kit

The complement system functional activity of the recombinant Sbi-III-IV-NS1 protein was assessed using Wieslab complement system screening kit (Euro Diagnostica, COMPL 300 RUO) in accordance with the enclosed manufacturer instruction. The assay system itself uses the principles of ELISA to quantitate the functional different pathways of complement activation in human serum by using labelled specific monoclonal antibodies that can detect the presence of neo-epitopes generated by complement activation. Microtitre plate wells are coated with specific lectin, classical or alternative pathway activator i.e. the LPS coated wells for the

alternative activation pathway, IgM coated wells for the classical activation pathway and mannan coated wells for the MBL activation pathway of complement.

In order to insure that only one pathway is activated in each assay, the diluents used in this assay contain pathway-specific blocker. For example, the alternative pathway diluent contains Ethylene-bis (oxyethylenenitrilo) tetraacetic acid (EGTA) that chelates the calcium required for the classical and the lectin pathway activation. The low serum concentration cannot activate the alternative pathway as it requires high serum concentration leaving the classical and the MBL pathways intact and in order to discriminate between them, the classical pathway is only activated on IgM coated strips in presence of divalent ions, calcium and magnesium. The MBL pathway is activated on mannan coated wells as the lectin pathway Mannan-Binding Lectin (MBL) recognition molecule binds to mannan and activated the complement system via the lectin pathway.

In order to investigate the possible roles of the recombinant Sbi-III-IV-NS1 protein in complement activation pathways, the recombinant protein was mixed with normal human serum to a final concentration of 0.5 mg/ml and incubated for 30 minutes at 30˚C. The mixture was then diluted 1:101 with the classical pathway and MBL pathway diluents (low serum concentration in a buffer containing calcium and magnesium), as provided with the kit, and 1:18 with the alternative pathway diluent (high serum concentration in a buffer containing magnesium and EGTA). After that, 100 µL of the diluted serum were added to the corresponding plate wells and 100 µL of the positive control (complement reference serum) and negative control sera provided were added to other wells. Wells received buffer only were considered as blank and plate incubated for one hour at 37°C. Plate was washed three times with 300 µL of washing solution followed by adding 100 µL of specific alkaline-phosphatase labelled antibody to a new antigen expressed in C5b-9 complex were added to each well and the plate was then incubated at room temperature for 30 minutes. After washing the plate 3 times, 100 µL of substrate solution was added to each well followed by incubation for 30 minutes at room temperature. The absorbance, correlated to complement activity, was measured at 405 nm using a microplate reader. The blank (diluent) absorbance was subtracted from samples, negative and positive control absorbance. The mean of pathway activated was

calculated from the formula [sample - negative control]/ [positive control - negative control] x100.

2.8 Circular dichroism (CD)

Circular dichroism (CD) is a useful method for determining the secondary folded structure of proteins and works by determining the asymmetry of molecules due to differences in absorption between left- and right- handed circular polarised light (71). In essence the CD signal between 190-250 nm and 250-350 nm can indicate characteristics such as a-helices and disulphide bonds allowing prediction of characteristics such as the presence of helices, b-sheet formation and coiling of the protein. Here CD experiments were performed on an Applied Photophysics Chiracsan instrument. The experiment was done in three scans at 20°C across a wavelength range of 200-300 nm and the path length was set at 1 mm and scanning intervals were 0.5 seconds. The protein sample concentration was 0.5 mg/ml in PBS.

2.9 Western blotting

Following expression of the pET28a plasmid containing the insert for the Sbi-III-IV-NS1 fusion protein in *E. coli* BL21, the bacteria were lysed and following SDS-PAGE and western blotting were performed to identify the Sbi-III-IV-NS1 recombinant protein. The SDS-PAGE separated proteins were transferred electrophoretically onto a nitrocellulose membrane at 25V for 12 minutes in transfer buffer. The membrane was then blocked with blocking buffer (5% skimmed milk in TBS) at room temperature, followed by washing the membrane twice with TBS/Tween-20 and the membrane was then incubated for 1h at RT with polyclonal rabbit anti-Sbi antibody [kind gift from Prof Timothy Foster, Trinity College Dublin] diluted 1:5000 in wash buffer. After that, the membrane was washed 3 times for 10 minutes each and incubated with HRP conjugated goat anti-rabbit IgG antibody (Thermo-Fisher, 815-968-0747) diluted 1:5000 in wash buffer for 1h. After this, the membrane was extensively washed (3 times for 10 minutes each). The

antibody was probed by using Pierce ECL western blotting substrate (Thermo Scientific). The last step was to expose the membrane to a light sensitive film before being developed and photographed.

Chapter Three: Results

3 Results

3.1 Expression and purification of recombinant Sbi-III-IV-NS1

The purpose of the project was to define and optimise a method for obtaining efficient expression of the hybrid protein Sbi-III-IV-NS1. The Sbi-III-IV-NS1 construct was expressed using the pET28a vector in *E. coli* BL21. This vector is commonly used as it incorporates a sequence that includes a repetitive histidine sequence (HisTag) to be incorporated at the N terminal of the transcribed recombinant protein and thus allows purification using nickel column affinity chromatography.

Sbi-III-IV-NS1 expression in *E. Coli* BL21 (DE3) was found to be present in inclusion bodies in all the conditions that were tried including different temperatures and different IPTG concentrations. It is quite common for the expression of recombinant proteins in *E. coli* to be found in inclusion bodies due to aggregation of the protein largely because prokaryotic cells do not glycosylate expressed proteins and these then become aggregated and form inclusion bodies where the protein is likely to be misfolded and inactive. Figure (6 A-B) shows SDS gel separation of the transformed bacterial proteins indicating that most of the proteins present accumulated as inclusion bodies.

The Sbi-III-IV-NS1 recombinant protein was purified by affinity chromatography using a HisTrap^{TM} HP column (GE Healthcare) which was operated using the AKTA purifier system. The sample was loaded onto a 1ml Hitrap column using the AKTA purifier (GE) and the loaded column was washed with 5 column volumes of HisA buffer and the bound Sbi-III-IV-NS1 fusion protein was eluted with HisB (20mM Tris, 300mM NaCl, 500mM imidazole) elution buffer. Figures 7 and 9 show the purification of Sbi-III-IV-NS1 using the AKTA purifier then judged by SDS gel (Figure 8).

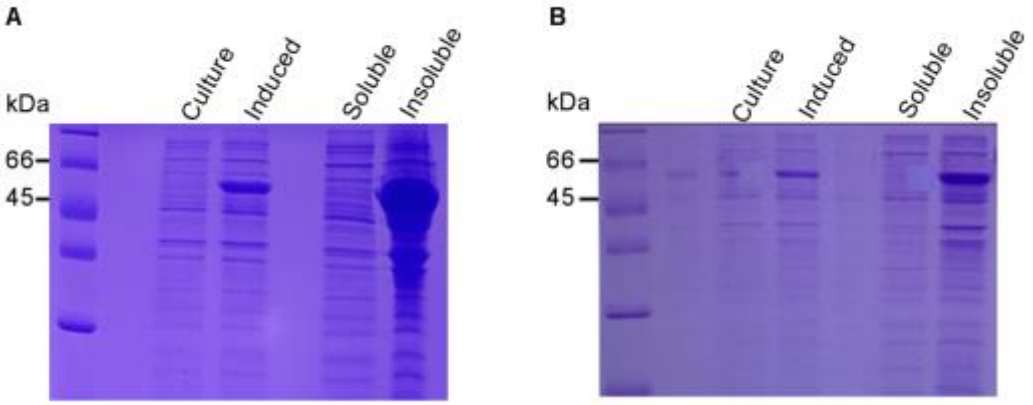

Figure 6: Expression trials for Sbi-III-IV-NS1

This figure shows a 12% SDS-gel stained with Coomassie blue of samples from *E. coli* expressing the Sbi-III-IV-NS1 fusion protein. The tracks include samples taken from the supernatant of un-induced cultures, supernatant from cultures induced with IPTG, bacterial extracts (after sonication) of the soluble fraction and extracts from the insoluble (bacterial pellet) fraction. The molecular weight ladder is shown in the left hand column. The transformed bacteria were cultured at both 25°C (A) and 16°C (**B).** In the induced cultures the presence of the recombinant Sbi-III-IV-NS1 protein at 55kDa.

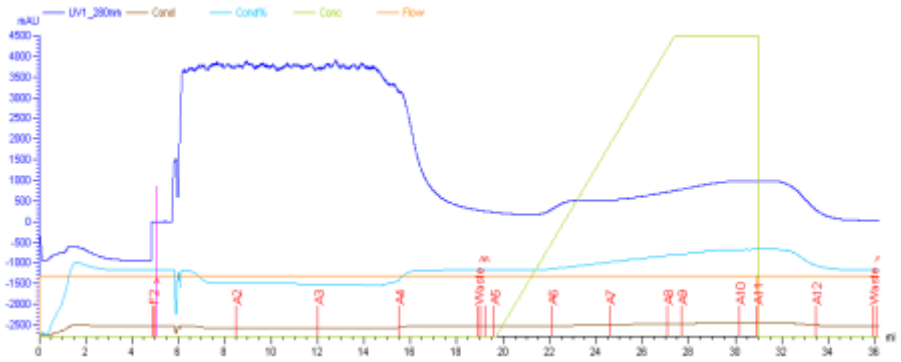

Figure 7: Purification of Sbi-III-IV-NS1 on AKTA affinity chromatography at 25°C

The Sbi-III-IV-NS1 recombinant protein was purified by affinity chromatography using a HisTrap™ HP column (GE Healthcare) which was operated using the AKTA purifier system. This system measures the conductivity and UV/Vis absorbance at 280 nm indicated as green or blue line, respectively. The blue line shows the absorbance at 280 nm. The green line shows the percentage of imidazole in the elution buffer (20mM Tris, 300mM NaCl, 500 mM imidazole PH: 7.4). Fractions were checked for the presence of Sbi-III-IV-NS1 by SDS-PAGE.

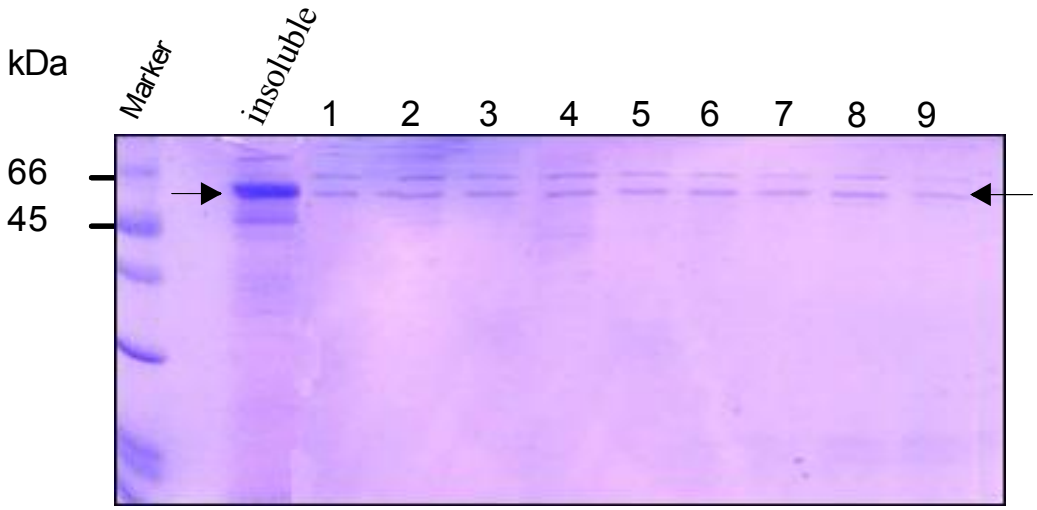

Figure 8: Expression trials for Sbi-III-IV-NS1

The figure shows the samples from the first 9 eluted fractions (Lanes 1-9 correspond to fractions A1-A9) following purification of the supernatant from *E. coli* expressing the Sbi-III-IV-NS1 fusion protein (Figure 6) using AKTA ion affinity chromatography at 25°C run on a 12% SDS gel stained with Coomassie blue. The insoluble pellet is shown in the first lane. The presence of the recombinant Sbi-III-IV-NS1 protein at 55kDa is shown to be readily present (indicated by arrow) in the insoluble pellet compared to the eluted fractions 1-9.

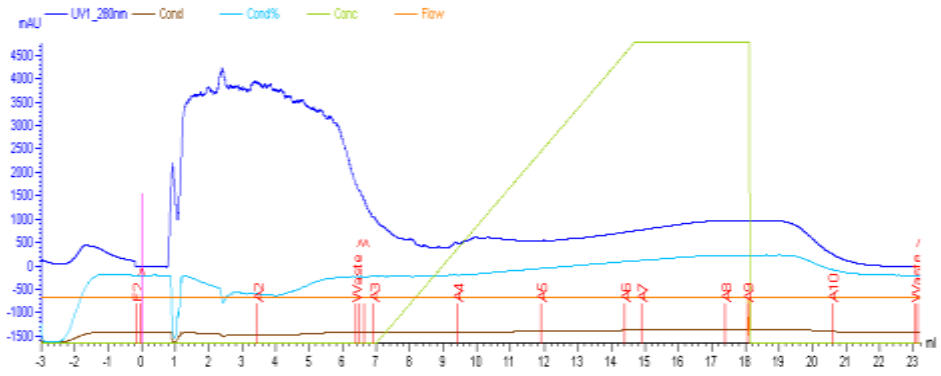

Figure 9: Purification of Sbi-III-IV-NS1 on AKTA ion affinity chromatography at 16°C

The Sbi-III-IV-NS1 recombinant protein was purified by affinity chromatography using a HisTrap™ HP column (GE Healthcare) which was operated using the AKTA purifier system. This system measures the conductivity and UV/Vis absorbance at 280 nm indicated as green or blue line, respectively. The blue line shows the absorbance at 280 nm. The green line shows the percentage of imidazole in the elution buffer (20mM Tris, 300mM NaCl, 500mM imidazole, PH: 7.4). Fractions were checked for the presence of Sbi-III-IV-NS1 by SDS-PAGE.

3.2 Expression of Sbi-III-IV-NS1 into Rosetta ™ 2(DE3)

Since expression of the recombinant Sbi-III-IV-NS1 protein was not obtained as a soluble protein using the *E. coli* BL21 strain, its derivative *E. coli* strain Rosetta™ was used which allows for more stable expression of eukaryotic proteins. The Rosetta strain contains plasmids that encode tRNAs for codons rarely used in *E. coli* thus providing a universal translation system. Hence, expression of the Sbi-III-IV-NS1 construct was attempted in Rosetta with the aim of obtaining recombinant protein in a native conformation (soluble) compared to the insoluble inclusion bodies obtained when the BL21 strain was used.

Expression in Rosetta cells (100 ml culture) was performed for 18 hours (overnight) and 10ml samples were collected at 4, 8 and 12 hours. Two 10ml samples were collected at 18 hours. Cells were lysed using sonication - however, one 10ml sample collected at 18 hours was treated with both sonication and the BugBuster lysis buffer to enhance lysis and release of expressed protein. No discernible difference was obtained with Rosetta cells at any timepoint, including the 18-hour sample treated with the BugBuster lysis buffer. Similar to expression with BL21 cells, the Sbi-III-IV-NS1 fusion protein was expressed as insoluble inclusion bodies at 24^{O}C (Figure 10).

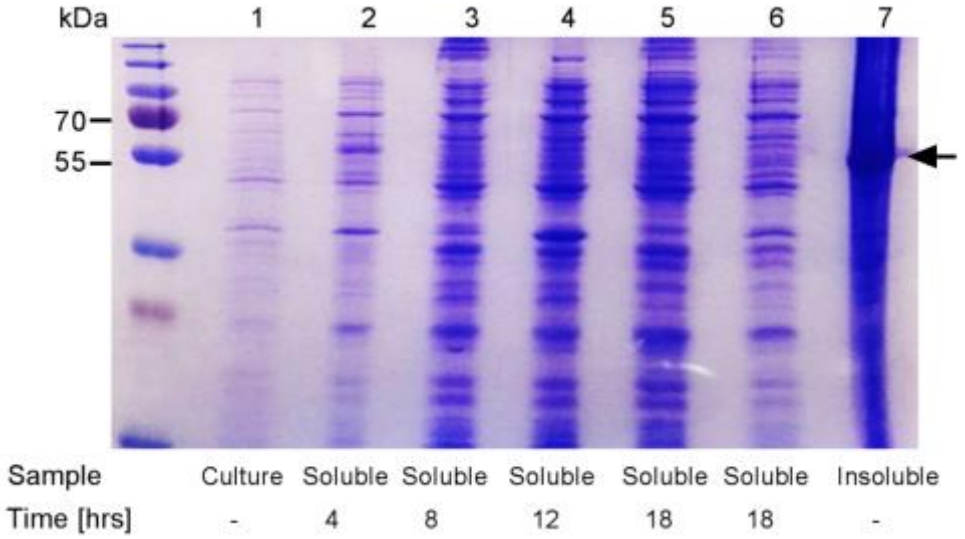

Figure 10: Expression of Sbi-III-IV-NS1 in Rosetta cells

This figure shows samples from *E. Coli* Rosetta cells expressing the Sbi-III-IV-NS1 fusion protein. The tracks show samples taken from the overnight induction with IPTG at different time points (4, 8, 12 and 18 hours) (lanes 2, 3, 4 and 5) which were then lysed by sonication and then run on a 12% SDS-PAGE gel and stained with Coomassie blue. The sample shown in Lane 6 was lysed by both the BugBuster buffer and by sonication. Lane 7 shows the extract of the sonicated cell pellet at 18 hours and lane 1 shows the culture supernatant. The molecular weight ladder is indicated to the far left and the arrows show the expected position of the Sbi-III-IV-NS1 fusion protein (55kDa).

3.3 Optimisation of the purification of recombinant Sbi-III-IV-NS1 from inclusion bodies using Nickel column affinity chromatography

Preliminary experiments had indicated that rather than being expressed as a soluble protein the recombinant Sbi-III-IV-NS1 protein was insoluble and accumulated in inclusion bodies within the *E. coli* cells. In order to optimise the purification the protein was solublised by using 8M urea and incubated in a series of different refolding buffers as mention in the methods section. Following HisGravi Trap affinity column purification the recombinant Sbi-III-IV-NS1 protein was eluted with PBS buffer containing 300 mM imidazole. The 15 eluted fractions from the different refolding buffer incubations were analysed by SDS-PAGE as shown in figure 11.

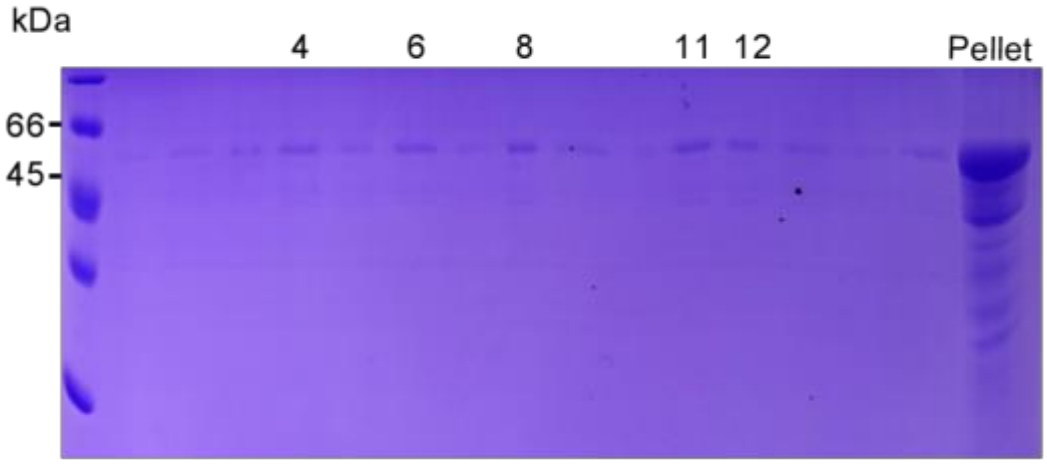

Figure 11: Screening of the expressed Sbi-III-IV-NS1 protein in different re-folding buffers

Following transfection of *E. coli* with the plasmid containing the Sbi-III-IV-NS1 insert, inclusion bodies were purified and exposed to a series of different refolding buffers from the Athena Refolding Kit and run on a12% SDS-gel which was stained with Coomassie blue. The gel shows the expected band size of Sbi-III-IV-NS1 (55kDa) and buffers 4, 8, 11 and 12 were more likely to be the optimal refolding buffers.

On the basis of the results shown in Figure 11 buffers 4, 8 and 12 were chosen to be used for refolding and further purification experiments of recombinant Sbi-III-IV-NS1 using its N-terminal histidine tag by affinity chromatography with a HisGravi Trap column (GE Healthcare) as shown in Figures 12, 13 and 14. These are the results of 3 separate experiments designed to optimise the imidazole (100-500 mM) concentration used for the elution from the HisGravi Trap column and the optimal refolding buffer.

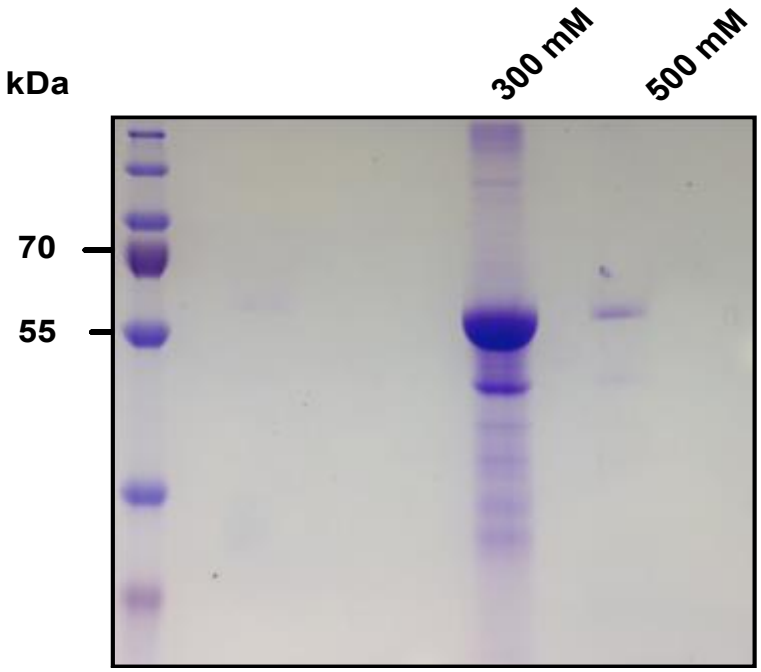

Figure 12: Purification of Sbi-III-IV-NS1 following incubation and refolding using buffer 4

A 12% SDS-gel was stained with Coomassie blue and shows bands of the elution fractions after purification with a HisGravi Trap column (GE Healthcare) using different elution concentrations of imidazole (300 and 500 mM). The gel shows a major band at the expected molecular weight of 55kDafollowing elution with 300mM imidazole.

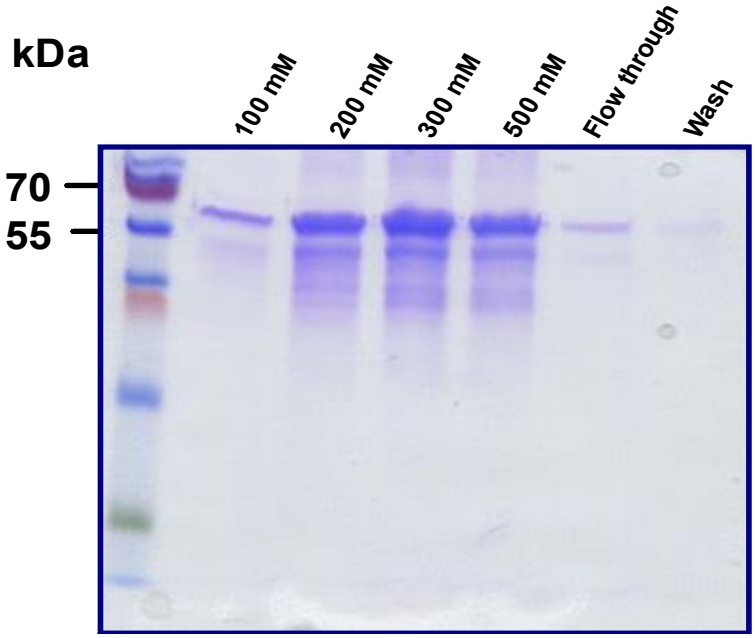

Figure 13: Purification of Sbi-III-IV-NS1 following incubation and refolding using buffer 8

A 12% SDS-gel was stained with Coomassie blue and shows bands of the elution fractions after purification with the HisGravi Trap column (GE Healthcare) using different elution concentrations of imidazole (100-500 mM) to optimise the elution conditions. The gel shows a major band at the expected molecular weight of 55kDa following elution with imidazole. F=flow through, W=wash.

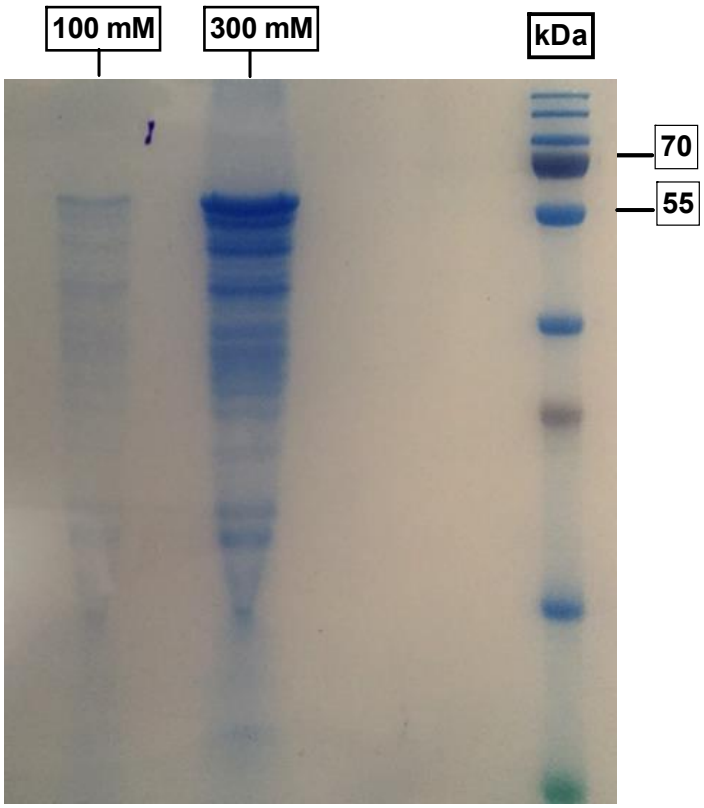

Figure 14: Purification of Sbi-III-IV-NS1 following incubation and refolding using buffer 12

A 12% SDS-gel was stained with Coomassie blue and shows bands of the elution fractions after purification with a HisGravi Trap column (GE Healthcare) using different elution concentrations of imidazole (100 and 300 mM). The gel shows a major band at the expected molecular weight of 55kDafollowing elution with 300mM imidazole.

3.4 Complement activation assay assessment using the Wieslab kit

In order to assess complement pathway activity (classical, alternative and MBL) in presence of Sbi-III-IV as part of the Sbi-III-IV-NS1 fusion protein, and its ability to deplete complement component C3 in Wieslab assay system (72), the soluble fusion protein obtained by refolding the protein isolated from inclusion bodies using buffer 12 of the Athena kit (Figure 14) was added to normal human serum and the mixture was then added to mannan, IgM and LPS coated wells to assay the MBL, the classical and the alternative pathway, respectively. No complement inhibition was detected in any of the three pathways in contrast to human serum (positive control) in which complement was shown to be fully active in all pathways (Figure 15 a, b and c).

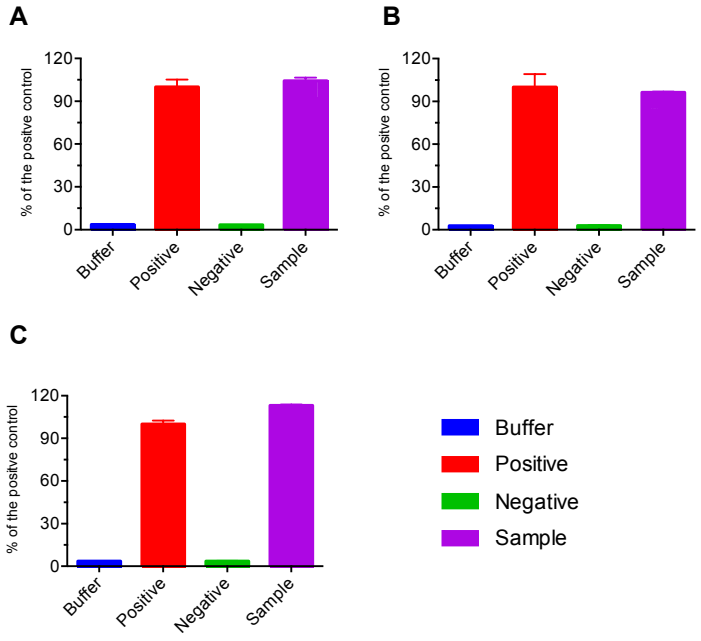

Figure 15: Functional activity assessment of the Sbi-III-IV-NS1 fusion protein in complement activation

The soluble and refolded Sbi-III-IV-NS1 fusion protein was mixed with human serum to a final concentration of 0.5 mg/ml. The mixture was the diluted to 1% in the classical and the MBL pathway diluent and added to IgM or mannan coated wells, respectively (A and C). For the alternative pathway assay, serum containing Sbi-III-IV-NS1 fusion protein was diluted to 5.5% in the alternative pathway diluent (B). The neo-antigens resulting from complement activation were probed with anti-C5b-9 antibody. The results show that there is no increase or decrease in the levels of complement activation in this assay comparable to normal human serum (positive control) indicating that this construct did not cause the depletion of complement component C3 as observed with Sbi-III-IV alone.

3.5 Circular dichroism (CD)

Since the Sbi-III-VI-NS1 contains an Sbi-IV domain, which is composed of three alpha-helices (73), it was expected that some degree of helicty will be observed in the CD data. The unstructured protein 1 (NS1) from Dengue virus is also known to contain significant beta-sheet structure (74), which would be expected to give a signal observable in the CD spectra in the region of 200-210 nm. The CD spectra (Figures 16 & 18), however show no evidence of helical structure in this region suggesting that the protein maybe present as a random coiled coil in solution because it is unfolded. These results could be simply due to poor signal to noise ratio in the region. The poor signal could be due to inaccuracy of measurement (protein concentration) or inadequate number of scans performed on the sample.

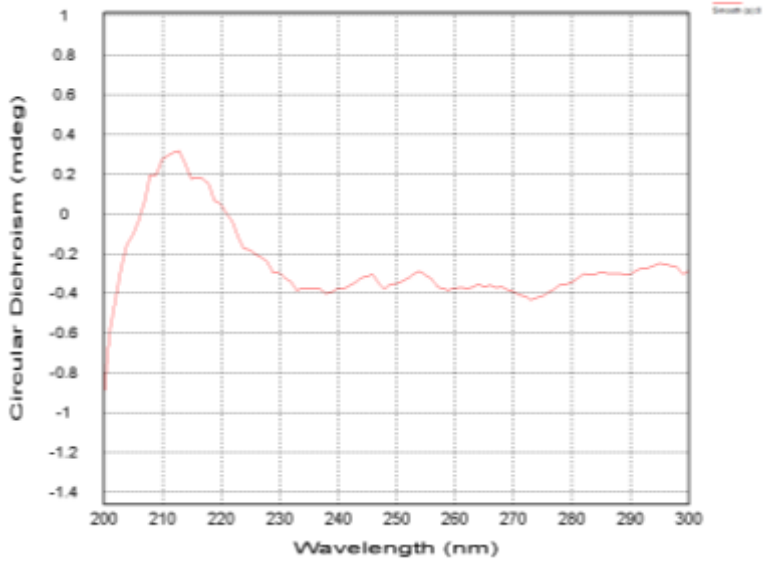

Figure 16: Smoothed data of 3 scans of the CD spectra of the Sbi-III-IV-NS1 fusion protein in PBS

No peaks were observed in the region scanned, suggesting that the fusion protein was in an unfolded state though a poor signal to noise ratio makes it difficult to make this claim conclusively

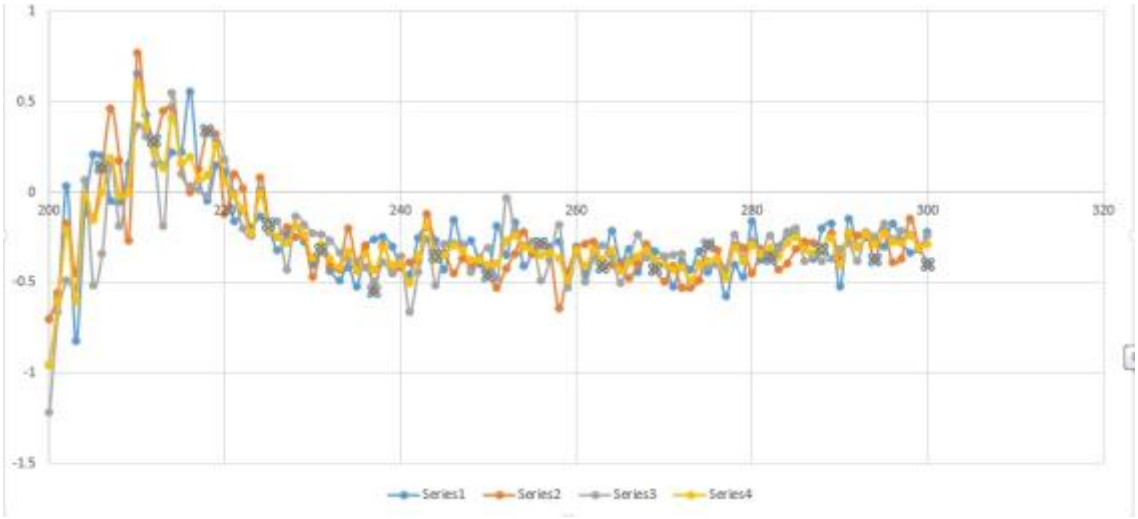

Figure 17: CD spectra of Sbi-III-IV-NS1 fusion protein in PBS

No significant alpha helicity observed in the region scanned, suggesting that the fusion protein was in an unfolded state. Series 1-3 represent replicates, series 4 represents an average of the three repeats. The data may improve by increasing the number of scans significantly.

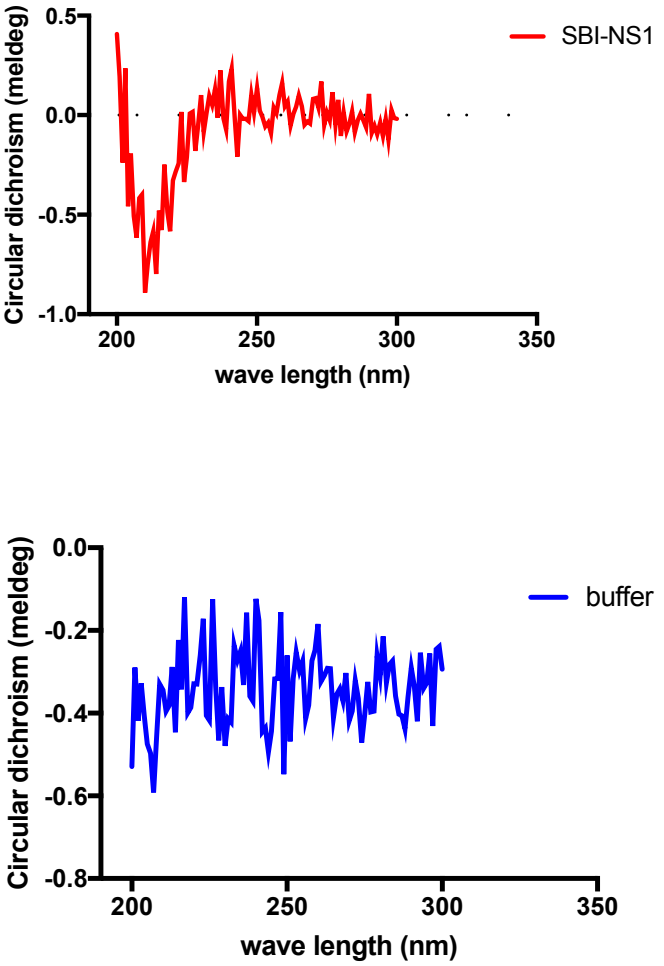

Figure 18: Smoothed data of 3 scans of the CD spectra of the Sbi-III-IV-NS1 fusion protein in PBS

The buffer spectrum (lower panel) was subtracted to ensure accuracy of the data.

3.6 Western blotting

Western blotting was performed to confirm the identity of the Sbi-III-IV-NS1 recombinant protein seen at 55 kDa in the SDS-PAGE experiments. After SDS-PAGE separated proteins were transferred electrophoretically onto a nitrocellulose membrane at 25V for 12 minutes in transfer buffer. Buffer 12 was used for refolding in this experiment. Figure 3-14 shows the Sbi-III-IV-NS1 recombinant protein on SDS-PAGE with the expected band size of 55 kDa (A) and (B) shows the western blot of the gel using polyclonal rabbit anti-Sbi antibody and probed with HRP conjugated goat anti-rabbit IgG antibody. The eluted proteins were thus confirmed as products of the recombinant plasmid.

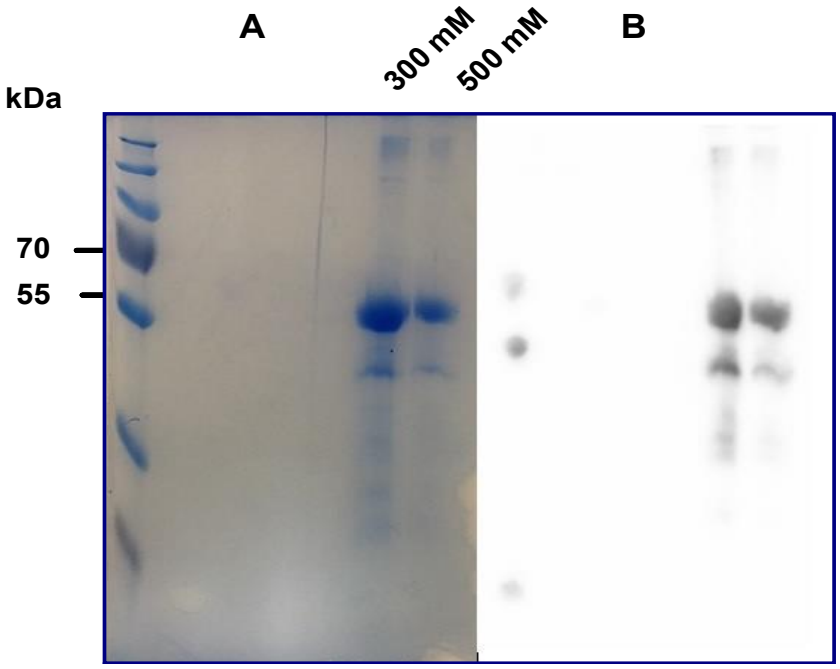

Figure 19: Western blot of Sbi-III-IV-NS1

A 12% SDS-gel was stained with Coomassie blue and shows bands of the elution fractions of refolding buffer 12, after purification with the HisGravi Trap column (GE Healthcare). The gel shows eluted fractions and shows a major band at the expected molecular weight of 55 kDa following elution with 300mM imidazole (A). Western blot analysis using polyclonal rabbit anti-Sbi antibody was used to confirm the identity of the eluted protein (B).

Chapter four: Discussion

4 Discussion

Dengue fever is a mosquito-borne tropical disease that affects millions of people, which when left untreated, causes mortality in about 50% of cases (1, 2). Dengue fever is caused by any of the 5 different serotypes of the dengue virus that have been identified. Infection with a specific (DENV) serotype confers lifelong immunity against that specific serotype but cross-protection against other serotypes lasts only a few months (10). Previous studies have shown that vaccine-mediated localised activation of complement factor C3 leads to improved pan-serotype immunological memory and opsonisation by phagocytes (59). Thus, the ideal DENV vaccine would contain a highly conserved immunogenic DENV protein conjugated to a complement activator. Therefore, this study aimed to construct a fusion protein comprising the highly-conserved DENV non-structural glycoprotein, NS1, and an immune evasion factor of *Staphylococcus aureus*, Sbi, which interacts with Factor H, C3b and C3b and C3d (75).

In order to achieve this aim the project set out to produce a recombinant protein that could both induce an immune response to the conserved DENV non-structural protein (NS1) and' in tandem, induce effective complement activation which would lead to highly efficient neutralisation and phagocytosis of infected cells and neutralise the infectious pathway.

Using a previously cloned hybrid Sbi-III-IV-NS1 gene cloned into the expression vector pET-28a, methods were optimised with *E.coli* BL21 and its derivative, Rosetta™, to obtain sufficient quantities of the recombinant hybrid protein to initiate vaccination strategies. However, although optimization of the expression conditions in *E. coli* allowed abundant quantities of recombinant Sbi-III-IV-NS1 to be produced (Figure 6) further experiments demonstrated that the expressed protein was not soluble and was found in inclusion bodies within the bacteria. This is not an uncommon finding in prokaryotic systems which do not allow appropriate glycosylation of eukaryotic derived proteins. In addition, the induction of the expression of the fusion protein with the prokaryotic promoter T7 and/or the absence of the appropriate eukaryotic cellular machinery in *E. coli* BL21 (and its derivative,

Rosetta^{TM}) required to maintain the fusion protein in its native state, may have led to the expression of the denatured fusion protein in inclusion bodies (76).

In order to try and isolate the expressed recombinant Sbi-III-IV-NS1 from the inclusion bodies, *E. coli* cells were first sonicated and the Sbi-III-IV-NS1 recombinant protein was first purified by affinity chromatography (Figures 7 and 9). However, no peaks of eluted proteins were observed and elution fractions run on SDS-PAGE showed no bands (Figure 8). This implied that no soluble proteins were present in the extract and the recombinant protein was effectively trapped in inclusion bodies, which has been described elsewhere (76).

Therefore, further experiments were performed in order to firstly release the denatured Sbi-III-IV-NS1 recombinant protein and, secondly, to solubilise it and allow the protein to refold into is native conformation.

Clearly stringent conditions were required to release and solubilise the denatured protein so the *E. coli* BL2 cultured bacteria were lysed and sonicated and the extract solubilised in 8M urea following which the protein concentration was adjusted to 1mg/ml and using the 'drip technique' was allowed to refold in a series of buffers designed to promote protein refolding as mention in the methods section. Following overnight incubation in the refolding buffer the Sbi-III-IV-NS1 recombinant protein was isolated using HisGravi Trap affinity column purification and conditions for optimal elution with imidazole was performed (Figures 12, 13 and 14).

Attempts to refold the protein from the inclusion bodies were not very successful with the best results being obtained when polyethylene glycol (PEG) [i.e. in buffer 4] was excluded from the isolation buffers, which could have enhanced the activity of Triton X-100, a non-ionic detergent that improves the solubility of proteins (Figures 12). However, circular dichroism experiments on the purified and refolded recombinant Sbi-III-IV-NS1 protein (Figure 16-18) were inconclusive. Circular dichroism is a method which allows the determination of the secondary refolded structure of the refolded recombinant Sbi-III-IV-NS1 protein but the Chirascan failed to register any protein helical structural motifs as would be expected from the published molecular models of Sbi-IV. In addition the non-structural protein 1

(NS1) from Dengue virus is known to contain significant beta-sheet structure (74) but again no signal was observed in the circular dichroism scan. This evidence therefore suggests that the numerous attempts to purify and appropriately refold the Sbi-III-IV-NS1 protein were ultimately unsuccessful.

While the clear advantage of using a mammalian expression system would be that proteins would be glycosylated and therefore remain stable and be more likely to remain in solution, it was thought that it might be preferable to use an *E. coli* expression system because of the need to produce sufficiently large amounts of the fusion protein that would allow testing in the *in vitro* assays that were established. Converse to mammalian expression systems involving cell lines such as the human embryonic kidney (HEK-293) and Chinese hamster ovary (CHO) cell lines, *E. coli* systems offer rapid and high expression of recombinant proteins. Furthermore, use of a mammalian expression system would have involved the generation of stable cell lines that would express the fusion proteins; a time-consuming process that could be complicated further by clonal variations and the need to keep the transgene stable under selection pressure.

Since the expression of soluble recombinant protein in *E. coli* can be influenced by different factors, such as temperature and IPTG concentration, in future studies, the expression of Sbi-III-IV-NS1 fusion protein will be assessed at different temperatures and lower (<1mM) IPTG concentrations in Rosetta™ cells. It is expected optimization of temperature and IPTG concentrations will increase yields of the native Sbi-III-IV-NS1 fusion protein. Expression of the individual components of the Sbi-III-IV-NS1 fusion protein separately, i.e. Sbi-III-IV by its own and NS1 alone, may be productive as a reduction in the complexity of the fusion protein may be responsible for its inability to fold correctly. Pooling of the individual proteins (Sbi-III-IV and NS1) in subsequent assays would then be attempted.

As NS1 is commonly secreted as an hexameric complex into the plasma of Dengue fever patients (63, 77) oxidation-dependent disulphide bond formation between the cysteine residues of the Sbi-III-IV-NS1 fusion protein (which possesses six potential disulphide bonds), is likely to be somewhat ineffective in the reductive cytoplasmic environment of *E. coli* (78). To counter the effect of reductases such as *trxB* (a

thioredoxin reductase) present in the cytoplasm, an *E. coli* strain containing the *trxB* mutation could be utilised. As the reductive activities of Thioredoxin-1 and -2 (encoded by *trxA* and *trxC* respectively) is dependent on their initial reduction by the gene product of *trxB*, only non-reduced forms of the thioredoxins will be present in the cytoplasm in a *trxB* mutant. These non-reduced thioredoxins can catalyse disulphide bond formation (79) thereby possibly promoting the stability of the recombinant Sbi-III-IV-NS1 fusion protein and its expression within the cytoplasm in a soluble form. This method has been successfully used to express sufficient amounts of cysteine-rich extracellular proteins such as the micronemal adhesive protein from *Toxoplasma gondii* (80). Obtaining functional isoforms of the fusion protein will therefore be tried using the *E. coli* trxB mutant expression system (commercially available as Origami™ 2(DE3)pLysS) or by targeting the expressed protein to the *E. coli* periplasm where disulphide bond-promoting enzymes abound to optimise appropriate folding of the protein.

In addition to using a strain with reduced reductase activity, co-expression of the Sbi-III-IV-NS1 protein with plasmids containing coding sequences for disulphide oxidoreductase and isomerase, DsbA and DsbC respectively, may ensure stability of the Sbi-III-IV-NS1 fusion protein as both DsbA and DsbC play crucial roles in the stability and functional expression of proteins *in vivo*(81, 82). Alternatively, the Sbi-III-IV-NS1 construct could be engineered to include a signal sequence that will target the expressed protein to the periplasm where the Dsb family of proteins is present.

In the Athena refolding kit experiments perhaps the optimal refolding was achieved with buffer 4 as judged by SDS-PAGE (Figure 12) and this could be confirmed with appropriate CD analysis. In future experiments detailed CD analysis of the Sbi-III-IV-NS1 protein should be undertaken to see whether the protein is expressed in its native confirmation. Further experiments might yield more definitive data if a higher concentration of protein and/or an increased number of scans were used to improve the signal to noise ratio.

Alternatively, the production of the fusion protein could be performed in the aforementioned mammalian expression systems (HEK-293 or CHO cell lines) or in baculovirus-infected insect cell lines (83). Despite the limitations mentioned previously, the key advantage of utilizing either insect or mammalian cell lines over the *E. coli* system would be the production of soluble recombinant proteins more likely to be expressed in their native conformation. These insect or mammalian systems provide glycosylation and other post-translational modifications, which could improve the solubility of the Sbi-III-IV-NS1 fusion protein. Exploiting the baculovirus vector system in lepidopteron cells and larvae (84) could be more productive since this system, termed MultiBac, affords the possibility of co-expressing the Sbi-III-IV and NS1 subunits in separate bacmids to yield the functional Sbi-III-IV-NS1 fusion protein complex. Following soluble protein expression, purification of the Sbi-III-IV-NS1 fusion protein secreted into growth media could be achieved using conventional monoclonal antibody-dependent immune-purification techniques (62) identified several monoclonal antibodies that bound to 3 highly-conserved epitopes of the NS1 protein. These antibodies, when covalently-bound to Protein G-matrices via crosslinking agents, could be used to isolate the soluble Sbi-III-IV-NS1 fusion protein from growth media.

Successful expression of the soluble Sbi-III-IV-NS1 fusion protein and its individual components (whose expression will be pursued as described previously) will pave the way for experiments in which the functional immunogenicity of the different proteins, i.e. their ability to induce neutralising antibodies, will be compared in mice (i.e. Sbi-III-IV-NS1 versus Sbi-III-IV and Sbi-III-IV-NS1 versus NS1).

In summary, an ideal vaccine for DENV will include antigenic components that are highly conserved among all described serotypes of DENV and can induce long-term immunological memory. The fusion protein described in this study satisfies vaccine candidacy as it contains the highly-conserved DENV non-structural protein NS1 and the Staphylococcal complement activator, Sbi. This fusion protein was successfully expressed in this study but not in a soluble, biologically-active state. To obtain functional isoforms of the fusion protein stability of the protein in the *E. coli* expression system should be pursued by utilising an *E. coli trxB* mutant (commercially available as Origami™ 2(DE3)pLysS) or by targeting the expressed

protein to the *E. coli* periplasm where disulphide bond-promoting enzymes can promote appropriate folding of the protein.

Chapter five: Summary and Conclusions

5 Summary, conclusions and future work

The purpose of the project was to develop a method for enhancing the development of effective immunity against Dengue Fever. It has previously been shown that the recruitment of C3d greatly enhances humoral immunity so therefore the aim of the project was to directly associate Sbi from staphylococcus (which activates the alternative complement pathway and indirectly recruits C3d) to NS1 a key target for immunoprotection against DENV. The aim of the project has therefore to express and produce sufficient quantities of Sbi-III-IV-NS1 for vaccine studies. However, firstly a number of hurdles had to be overcome, notably determining the optimal conditions for Sbi-III-IV-NS1 expression using different *E. coli* strains and different culture conditions including, time, temperature and IPTG concentration. Secondly it became clear that the Sbi-III-IV-NS1 protein was not present in soluble extracts of the transformed *E. coli* BL2 bacteria and that the Sbi-III-IV-NS1 recombinant protein was expressed in a denatured form in inclusion bodies. A number of methods were then used to optimize the isolation and the appropriate refolding of the Sbi-III-IV-NS1 recombinant protein which were assessed by SDS-PAGE and CD. Although significant progress was made there was insufficient time to progress to the next stage of the project which was to use the purified Sbi-III-IV-NS1 protein to assess its ability to produce resistance to DENV (in mice) using the construct to recruit C3d which should enhance humoral immunity to the virus and pave the way for future vaccine based experiments.

Future directions

Firstly, future studies could assess the expression of the Sbi-III-IV-NS1 fusion protein at different temperatures and lower (<1mM) IPTG concentrations in RosettaTM cells as well as trying to express the individual components Sbi-III-IV and NS1 separately as the complexity of the fusion protein may contribute to its lack of solubility and inability to fold appropriately.

Secondly, as refolding of this recombinant protein is dependent of the formation of a number of disulphide bonds the effect of reductases such as *trxB* (a thioredoxin reductase) present in the cytoplasm, an *E. coli* strain containing the *trxB* mutation could be utilized. In addition, plasmids containing coding sequences for disulphide oxidoreductase and isomerase may improve the stability of the Sbi-III-IV-NS1 fusion protein or, alternatively, the Sbi-III-IV-NS1 construct could be engineered to include a signal sequence that will target the expressed protein to the periplasm where the Dsb family of proteins is present.

Finally, if ssuccessful expression of a soluble form of the Sbi-III-IV-NS1 fusion protein and its individual components can be achieved it will then be possible to study their ability to induce neutralising antibodies in mouse model systems.

Chapter six: References

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Chapter seven: Appendices

7 Appendices

7.1 Appendix 1

7.1.1 Sbi-III-IV-NS1 conjugate.

The coding sequence of Sbi domains 3 and 4 was cloned into the pET28a (Kan^r)vector using the *Nhe*I and *BamH*I restriction sites and, in addition, the coding sequence of Dengue virus NS1 was inserted using the *BamH*I and *Xho*I restriction sites to make a plasmid construct that could be expressed in *E. coli*. The recombinant Sbi-III-IV-NS1 carried an N-terminal His-tag for protein purification. (This work was performed by Dr Gyles Cozier). Sbi-III-IV **is in red.** NS1 is in blue.

(HisTag) (Thrombin)
ATG GGC AGC AGC **CAT CAT CAT CAT CAT CAC** AGC AGC GGC **CTG GTG CCG CGC GGC AGC** CAT
M G S S H H H H H H S S G L V P R G S H
(*Nhe*I)
ATG **GCT AGC** GAA CGT CAA AAT ATT GAA AAT GCG GAT AAA GCA ATT AAA GAT TTC CAA GAT
M **A S** E R Q N I E N A D K A I K D F Q D

AAC AAA GCA CCA CAC GAT AAA TCA GCA GCA TAT GAA GCT AAC TCA AAA TTA CCT AAA GAT
N K A P H D K S A A Y E A N S K L P K D

TTA CGC GAT AAA AAT AAC CGC TTT GTA GAA AAA GTT TCA ATT GAA AAA GCA ATC GTT CGT
L R D K N N R F V E K V S I E K A I V R

CAT GAT GAG CGT GTG AAA TCA GCA AAT GAT GCA ATC TCA AAA TTA AAT GAA AAA GAT TCA
H D E R V K S A N D A I S K L N E K D S

ATT GAA AAC AGA CGT TTA GCA CAA CGT GAA GTT AAC AAA GCA CCT ATG GAT GTA AAA GAG
I E N R R L A Q R E V N K A P M D V K E

CAT TTA CAG AAA CAA TTA GAC GCA TTA GTA GCT CAA AAA GAT GCT GAA AAG AAA GTG GCG
H L Q K Q L D A L V A Q K D A E K K V A
(*BamH*I)
GGA TCC GAC AGT GGT TGC GTT GTG AGC TGG AAG AAC AAA GAA CTG AAA TGC GGC AAT GGG
G S D S G C V V S W K N K E L K C G N G

ATA TTT GTC ACA GAT AAC GTG CAT ACA TGG ACA GAA CAA TAC AAG TTC CAA CCA GAA TCC
I F V T D N V H T W T E Q Y K F Q P E S

CCT TCA AAA CTG GCT TCA GCT ATT CAG AAA GCT CAT GAA GAG GGC ATT TGT GGA ATC CGC
P S K L A S A I Q K A H E E G I C G I R

TCA GTA ACA AGA CTG GAA AAT CTT ATG TGG AAA CAG ATA ACA CCG GAA TTG AAC CAC ATC
S V T R L E N L M W K Q I T P E L N H I

CTA TCA GAA AAT GAA GTG AAG CTG ACT ATC ATG ACA GGA GAC ATT AAA GGA ATC ATG CAG
L S E N E V K L T I M T G D I K G I M Q

GTA GGA AAA CGA TCT CTG CAG CCC CAA CCC ACT GAG CTG AGG TAT TCA TGG AAA ACA TGG
V G K R S L Q P Q P T E L R Y S W K T W

GGT AAA GCA AAA ATG CTC TCC ACA GAA CTC CAC AAC CAG ACC TTC CTT ATT GAT GGT CCC
G K A K M L S T E L H N Q T F L I D G P

GAA ACA GCA GAA TGC CCC AAC ACA AAC AGA GCT TGG AAT TCA CTG GAA GTT GAG GAC TAT
E T A E C P N T N R A W N S L E V E D Y

GGC TTT GGA GTA TTC ACC ACC AAT ATA TGG CTA AAA TTG AGA GAG AAG CAG GAT GTA TTT
G F G V F T T N I W L K L R E K Q D V F

TGT GAC TCA AAA CTT ATG TCA GCG GCC ATA AAG GAC AAC AGA GCC GTC CAT GCT GAT ATG
C D S K L M S A A I K D N R A V H A D M

GGT TAT TGG ATA GAA AGT GCA CTC AAT GAC ACA TGG AAG ATA GAA AAA GCC TCT TTC ATT
G Y W I E S A L N D T W K I E K A S F I

GAA GTT AAA AGT TGC CAC TGG CCA AAG TCA CAC ACT CTT TGG AGC AAT GGA GTG CTA GAA
E V K S C H W P K S H T L W S N G V L E

AGT GAG ATG ATA ATT CCA AAG AAT CTC GCT GGA CCA GTG TCA CAA CAC AAT AAC AGA CCA
S E M I I P K N L A G P V S Q H N N R P

GGC TAT TAC ACA CAA ACA GCA GGA CCT TGG CAT CTA GGC AAA CTT GAG ATG GAC TTT GAT
G Y Y T Q T A G P W H L G K L E M D F D

TTC TGC AAA GGA ACT ACA GTG GTG GTG ACC GAG GAC TGT GGA AAC AGA GGA CCC TCT TTA
F C K G T T V V V T E D C G N R G P S L

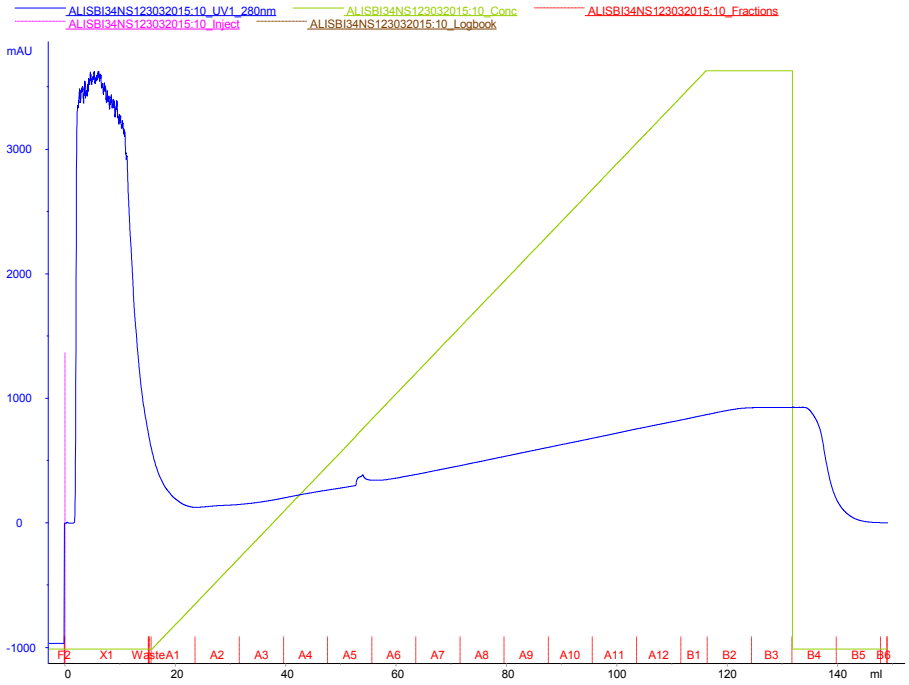
AGA ACA ACC ACT GCC TCT GGA AAA CTT ATA ACG GAA TGG TGC TGT CGA TCT TGC ACA TTG
R T T T A S G K L I T E W C C R S C T L

CCA CCG TTA AGA TAC AGA GGT GAG GAT GGA TGC TGG TAT GGG ATG GAA ATT AGA CCA TTG
P P L R Y R G E D G C W Y G M E I R P L

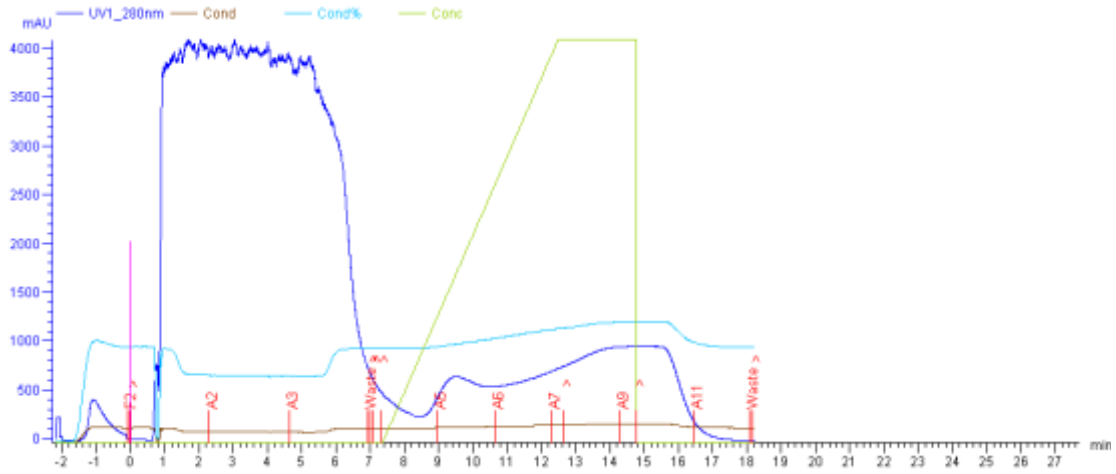
AAA GAG AAA GAA GAA AAT CTG GTC AAC TCC TTG GTC ACA GCC **STOP**
K E K E E N L V N S L V T A

7.2 Appendix 2

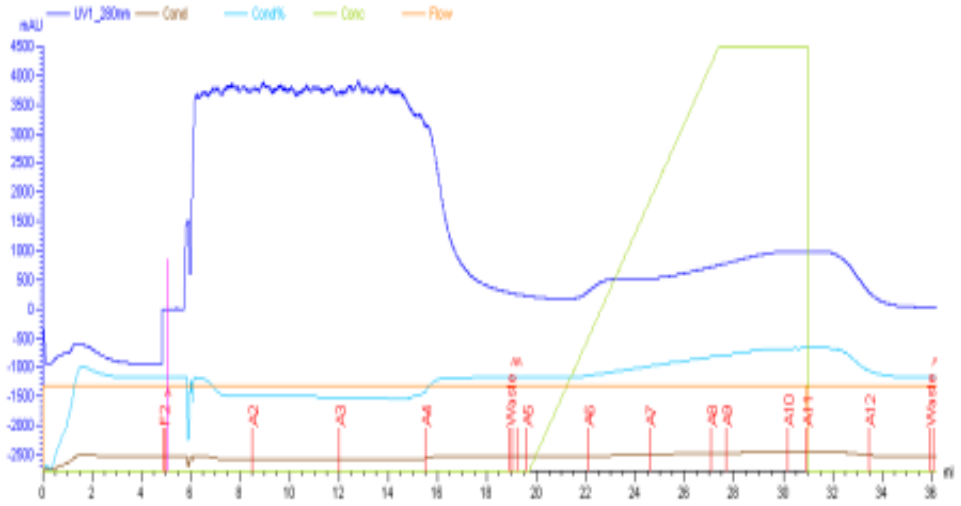
The purification of expressed Sbi-III-IV-NS1 by AKTA affinity chromatography using different conditions.



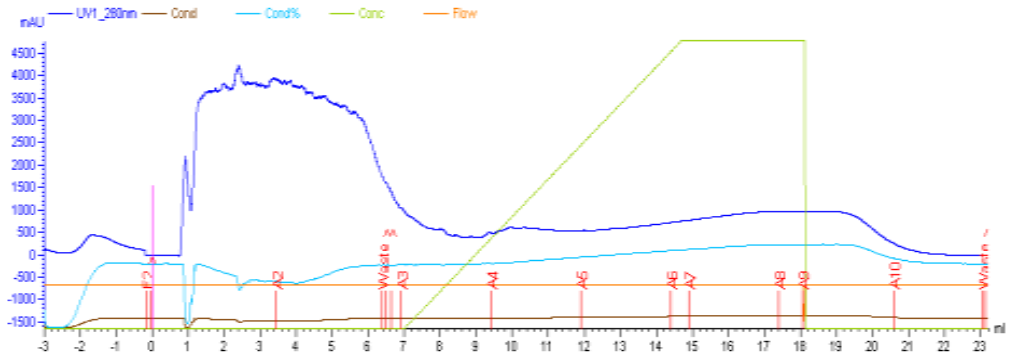
Purification of Sbi-III-IV-NS1 by AKTA affinity chromatography at 12°C



Purification of Sbi-III-IV-NS1 by AKTA affinity chromatography at 16°C



Purification of Sbi-III-IV-NS1 by AKTA affinity chromatography at 20°C



Purification of Sbi-III-IV-NS1 by AKTA affinity chromatography at 25°C

7.3 Appendix 3

7.3.1 Buffers and solutions

7.3.1.1 Protocol to process Inclusion body pellets

Solubilisation buffer	(25 mM Tris-HCL pH 7.5, 8 M Urea with 5 mM DDT at pH 8) 50 ml

1	Lysis buffer (lysosome)	(25mM Tris-HCL pH 8.0, 150 mM NaCl, 0.5 mg/ml lysozyme, 1 mM EDTA) 50 ML
2	Wash buffer (Triton)	(25 mM Tris-HCL pH 8.0, 0.5 M NaCL, 0.5% Triton X-100, 1 mM EDTA) 50 ML
3	Wash buffer (Urea)	(25mM Tris-HCL pH 8.0, 0.5 M NaCl, 1 mg/ml Sodium deoxycholic, 1 M Urea) 50 ML

7.3.1.2 Refolding Buffers from the Athena kit

Buffer 4	50 mM MES pH 6.0, 240 mM NaCl, 10 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 0.5 M arginine, 0.5% Triton X-100, 1 mM GSH, 0.1 mM GSSH
Buffer 6	50 mM MES pH 6.0, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3550, 1 mM GSH, 0.1 mM GSSH
Buffer 8	50 mM Tris-Cl pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3550, 1 mM GSH, 0.1 mM GSSH
Buffer 12	50 mM Tris-Cl pH 8.5, 240 mM NaCl, 10 mM KCl, 1 mM EDTA,

	0.05% polyethylene glycol 3550, 1 mM GSH, 0.1 mM GSSH

7.3.1.3 Akta Purification buffers

His-A (washing)	20mM Tris + 300mM NaCl +20mM imidazole **pH 7.4**
His-B (elution)	20mM Tris + 300mM NaCl +500mM imidazole **pH 7.4**

7.3.1.4 TBS buffer

7.3.1.5 SDS-PAGE

7.3.1.5.1 Separating gel 12%

Tris (ph8)	1.5625 ml
Acrylamide	1.867 ml
Water	2.77 ml
TEMED	2.8125 ul
APS 10%	31.25 ul

7.3.1.5.2 Staking gel

0.5 M Tris (pH6.8)	0.7813 ml

Acrylamide	0.3125 ml
Water	2 ml
TEMED	3.125 ul
APS 10%	15.625 ul

7.4 Appendix 4

7.4.1 Media

7.4.1.1 Luria broth

10 gm	Bacto Tryptone
5 gm	Yeast extract
10 gm	NaCl
Total volume	1000 ml

7.4.1.2 Stock Solution Preparation of 1 M IPTG

2.38 gm	IPTG
5 gm	Yeast extract
Filter	0.22 μ syringe filter
Total final volume	10 ml